NOV 12 1930

III Medical Lib. Number 4

Physiological Zoölogy

OCTOBER, 1930

e Relation between the Resistance to Heat and the Mechanism of th in *Paramecium* - - - - - - - - - - *H. W. Chalkley* 425

High Toxicity of Nascent Oxygen - - - - - *Carl L. Hubbs* 441

of Experimental Hyperthyroidism on Reproductive Processes of ale Albino Rats - - - - - - - - - *Charles K. Weichert* 461

ydew Reflexes - - - - - - - - - - - *E. A. Andrews* 467

dy of Chitin Secretion in *Daphnia magna*
B. G. Anderson and L. A. Brown 485

ythemia in White Rats
L. B. Nice, Mary Elizabeth Morris, and W. P. Elhardt 494

Development. I. Fatty Acids in the Grasshopper Egg
Eleanor H. Slifer 503

titative Study of the Effect of Chloroform on Oxygen Consumption Tadpole (*Rana clamitans*) - - - - - - *Sidney A. Narins* 519

e Development of *Arbacia* Eggs in Cyanides *Reuben Blumenthal* 539

to Volume III - - - - - - - - - - - - - - 565

THE UNIVERSITY OF CHICAGO PRESS
CHICAGO, ILLINOIS, U.S.A.

PHYSIOLOGICAL ZOÖLOGY

A Quarterly Journal of Zoölogical Research

CHARLES MANNING CHILD AND WARDER C. ALLEE
Managing Editors

EDITORIAL BOARD

M. F. Guyer, *University of Wisconsin*
L. V. Heilbrunn, *University of Pennsylvania*
E. E. Just, *Howard University*
F. R. Lillie, *University of Chicago*
R. S. Lillie, *University of Chicago*
W. W. Swingle, *Princeton University*
C. V. Taylor, *Stanford University*
J. H. Bodine, *University of Iowa*

Physiological Zoölogy is published quarterly by the University of Chicago at the University Press, 5750 Ellis Avenue, Chicago, Ill. The subscription price is $7.50 a year; the price of single copies is $2.25. Orders for service of less than a half-year will be charged at the single-copy rate. Postage is prepaid by the publishers on all orders from the United States, Mexico, Cuba, Porto Rico, Panama Canal Zone, Republic of Panama, Dominican Republic, Canary Islands, El Salvador, Argentina, Bolivia, Brazil, Colombia, Chile, Costa Rica, Ecuador, Guatemala, Honduras, Nicaragua, Peru, Hayti, Uruguay, Paraguay, Hawaiian Islands, Philippine Islands, Guam, Samoan Islands, Balearic Islands, Spain, and Venezuela. Postage is charged extra as follows: for Canada and Newfoundland, 15 cents on annual subscriptions (total $7.65), on single copies 4 cents (total $2.29); for all other countries in the Postal Union, 25 cents on annual subscriptions (total $7.75), on single copies 6 cents (total $2.31). Patrons are requested to make all remittances payable to The University of Chicago Press in postal or express money orders or bank drafts.

The following are authorized agents:

For the British Empire, except North America, India, and Australasia: The Cambridge University Press, Fetter Lane, London, E.C. 4, England. Yearly subscriptions, including postage, 35*s.* 2*d.* each; single copies, including postage, 10*s.* 10*d.* each.

For Japan: The Maruzen Company, Ltd., Tokyo.

For China: The Commercial Press, Ltd., Paoshan Road, Shanghai. Yearly subscriptions, $7.50; single copies, $2.25, or their equivalents in Chinese money. Postage extra on yearly subscriptions 25 cents, on single copies 6 cents.

Claims for missing numbers should be made within the month following the regular month of publication. The publishers expect to supply missing numbers free only when losses have been sustained in transit and when the reserve stock will permit.

Business correspondence should be addressed to The University of Chicago Press, Chicago, Ill.

Communications for the editors and manuscripts should be addressed to W. C. Allee, Managing Editor of Physiological Zoölogy, The University of Chicago, Chicago, Ill.

The articles in this journal are indexed in the *International Index to Periodicals*, New York, N.Y.

Entered as second-class matter October 27, 1928, at the post-office at Chicago, Illinois, under the Act of March 3, 1879.

[PRINTED IN U.S.A.]

Physiological Zoölogy

Vol. III OCTOBER 1930 *No. 4*

ON THE RELATION BETWEEN THE RESISTANCE TO HEAT AND THE MECHANISM OF DEATH IN PARAMECIUM[1]

(Four figures)

H. W. CHALKLEY
Division of Pharmacology, Hygienic Laboratory, U.S. Public Health Service, Washington, D.C.

INTRODUCTION

IT IS well known that coagulative changes play a fundamental rôle in the heat death of cells; but the exact mechanism by which the actual death of the cell results has not, as far as the writer is aware, been ascertained. This would appear to have considerable interest, inasmuch as the way in which the living mechanism fails should throw considerable light on the function of its parts and should make it possible to obtain a significant, if fleeting, analysis of their normal relations.

In the course of experiments in this laboratory by Kahler, Chalkley, and Voegtlin (1929) on the effect of a high-frequency electric field on *Paramecium*, it was noted that organisms from different cultures differed slightly in their resistance to heat. It was also noted that death in individual cases occurred sometimes by swelling and bursting of the cell and sometimes apparently by coagulation. These two facts suggested that the environmental conditions played an important part in determining the resistance and that the actual mechanism of death differed under different conditions.

---

[1] The problem originated at the instigation of Professor Carl Voegtlin, chief of the Division of Pharmacology. The author wishes to express his thanks for his advice and suggestions during the progress of the investigation.

Experiments were initiated to ascertain if environmental changes did in fact influence resistance. The results reported in a recent publication (Chalkley, 1930) showed that changes in both hydrogen-ion concentration and salt balance in the surrounding medium were effective factors. However, in this publication no attempt was made to analyze the mechanism involved. The present report represents an attempt at such analysis, using the previously reported data, which are here recapitulated for the convenience of the reader.

MATERIAL AND METHODS

Since material was abundant, since its unicellular character made rapid and complete environmental change a simple matter, and since changes in its normal motility would serve as an excellent index of injury, *Paramecium caudatum* was used in these experiments. The *Paramecia* utilized were the progeny of a single cell.[1] Culture was made in glass crystallizing dishes, in saline solution composed of NaCl 0.1 gram; $CaCl_2$ 0.004 gram; KCl 0.004 gram; $NaHCO_3$ 0.004 gram; H_2O 1,000 cc. From ten to twelve wheat grains were added to each culture. The hydrogen-ion concentration of the cultures was maintained at slight acidity, pH 6.8 to 6.2, by addition of food (wheat) when necessary. Closer control was found to be unnecessary.

The saline solutions in which the *Paramecia* were heated were: (1) saline as used for culture, referred to hereafter as balanced saline;[2] (2) the same but with the KCl omitted and $CaCl_2$ added in equimolecular concentration; (3) the same but with the $CaCl_2$ omitted and KCl added in equimolecular concentration; (4) the same but with KCl and $CaCl_2$ omitted and NaCl added in equimolecular concentration. All of these were used at hydrogen-ion concentrations from pH 5.8 or 6.0 to 8.4 or 8.6 as the continued resistance of the organisms required. The pH of all solutions was controlled as follows: The solution was adjusted to a point slightly alkaline of the required value by omitting a portion of the NaCl and substituting $NaHCO_3$, the substitution being made so that the Na concentration was not changed. The exact pH wanted was then reached by addition of dilute HCl with aëration to remove CO_2 until the solution was stable at the requisite pH over a period of at least 2 hours. The error due to dilution with HCl was deemed negligible, as it never exceeded 0.2 per cent volume. The pH measurements were made with a La Motte colorimetric set; and since with these slightly buffered solutions the indicator might introduce error, two indicators at least were used for each determination. The indicators used were methyl red, bromthymol purple, thymol blue, phenol red, and cresol red. These allow simultaneous colorimetric measurements with two different indicators over the range used, with the exception of pH 8.6, where cresol red was used alone. All solutions were found to be nontoxic at room temperature over a period of 48 hours with the exception of the solutions containing Na alone. These were slightly toxic, dead organisms appearing in 8–12 hours.

---

[1] No conjugation was observed during the course of the investigation.

[2] The term "balanced" is not used to imply a physiologically determined optimum condition. The solution used is simply one that was found empirically to be suitable for culture purposes.

The experimental procedure throughout was as follows: Ten cc. of culture fluid with suspended *Paramecia* were taken from a vigorous culture. The *Paramecia* thus obtained were washed thrice with balanced saline at pH 6.8 by low-speed centrifugation, and then left for 24 hours. From these *Paramecia* lots of twenty-five each were removed with a paraffined capillary pipette and placed separately in a paraffined Petrie dish. Then, by means of a pipette, superfluous saline was removed until the drop containing the *Paramecia* was reduced to approximately 0.02 cc., as checked by comparison with measured drops of saline placed in the dish for reference. Except when under manipulation, the dish was kept covered to prevent evaporation or accidental contamination.

After this the lots of *Paramecia* were transferred separately at timed intervals to paraffined test tubes each of which contained 5 cc. of the saline solution used. These tubes had all been previously brought to 40° C. in a water bath and were maintained at that temperature throughout the experiment.[1] When the exposure to heat was completed, all tubes were removed, simultaneously, from the bath and were cooled in a second bath at 20°–22° C. for 30 minutes. At the end of this time the tubes were emptied separately into small round-bottomed glass dishes, and the surviving *Paramecia* in each lot counted. Each individual as counted was removed from the dish with a pipette. A test of the accuracy of count was made by running through the procedure with the baths at room temperature. Ten such runs gave a maximum error occurring once in the ten runs, of two *Paramecia*, or 8 per cent on individual counts. A binocular dissecting microscope giving magnification to 30× was used for counting and observation.

---

[1] Temperature control was secured to 0.1° C. by means of a De Khotinksky thermostat.

THE EFFECT OF CHANGES IN HYDROGEN-ION CONCENTRATION ON THE RESISTANCE OF PARAMECIA TO A TEMPERATURE OF 40° C. WHEN EXPOSURE IS MADE (A) IN BALANCED SALINE; (B) IN SALINE WITH POTASSIUM EXCESS; (C) IN SALINE WITH CALCIUM EXCESS; (D) IN SALINE WITH SODIUM EXCESS

A. *Paramecia* were exposed to 40° C. in balanced saline at pH 6.0 to 8.6 in 0.2 unit steps, for 2, 4, 8, 12, and 16 minutes. Five counts were obtained of the number of Protozoa surviving at the end of each time interval in each hydrogen-ion concentration used. These counts were averaged and the percentage of survivors plotted as a function of time. This gave a series of curves which showed the rate of death at each pH. From this series of curves the time taken to kill 50 per cent at each pH was obtained by interpolation and plotted as a function of pH. Inasmuch as the making of this series of tests took several days, controls were established by obtaining, after all the counts were completed for a given pH, a single count at pH 6.6 (the first pH value used) at each time interval used, for comparison with the previous counts. Similarly, when other solutions were used, such control counts were always made using the balanced solution at pH 6.4, 6.6, or 7.0. The latitude in pH value was allowed because, owing to the difficulty of making the solutions, it seemed impracticable to run all controls at exactly the same pH. Further, once the curve for the balanced solution was established, variation in the pH used for controls served to establish the curve in the balanced solution more firmly by providing additional check points. The number of cells coagulated, i.e., in which the protoplasm was opaque and no rupture of the cells had occurred, was noted in all counts. The figures obtained were averaged for each pH step and plotted as percentages of total deaths for each pH. Also, for each pH a qualitative observation was made of the swelling of the cell. This observation was based on simultaneous ocular comparison of surviving *Paramecia* with normal individuals. The curves obtained are presented

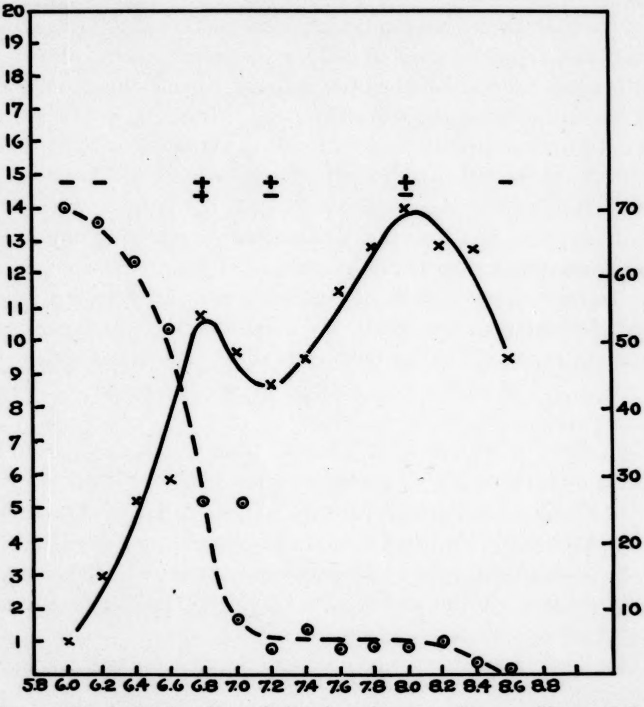


FIG. 1.—Graph showing the variations in resistance to heat, swelling, and the percentage of deaths due to coagulation of the cell, in Paramecia, exposed to 40° C. in balanced saline at different hydrogen-ion concentrations. Abscissas, pH; ordinates, scale on left, time in minutes to kill 50 per cent; scale on right, percentage of deaths in which cells were unruptured and coagulated; continuous line, resistance; broken line, percentage of deaths by coagulation. Swelling indicated as follows: —none or shrinkage; ±slight to none; +marked; ‡great.

in Figure 1. From the curves it will be noted that the time of resistance rises from 1 minute at pH 6.0 to a maximum of 10.6 minutes at pH 6.8, drops to a minimum of 8.8 minutes at pH 7.2, rises again to a maximum of 14 minutes at pH 8.0, and then declines to 9.5 minutes at pH 8.6. The percentage of cells coagulated is 70 per cent at pH 6.0. It then drops gradually to about 5 per cent at pH 7.2, remains relatively constant at pH 8.2, and then drops gradually to 2 per cent at pH 8.6.

Swelling apparently is negligible or absent at pH 6.0 but great at pH 6.8, and apparently less at pH 7.0 and on into the alkaline range. Between pH 6.8 and 8.0 the cells that did not die from coagulation died from rupture of the membrane due to swelling of the cell contents. In the extreme alkaline range from approximately pH 8.0 on, few fragments of cells were found. The membrane and contents dissolved rapidly. The degree of swelling as noted in surviving cells was, if anything, less than that at neutrality; and death appeared to result from dissolution of the membrane rather than its rupture.

B. In this experiment the procedure was as previously outlined, except that exposure was made in a saline solution which contained, in place of the $CaCl_2$ of the balanced saline, an equimolecular addition of KCl. The curves for variation of resistance and coagulation are presented in Figure 2.

From these it will be seen that the time of resistance increases from 2.4 minutes at pH 5.8 to 9.5 minutes at pH 6.0, declines gradually to about 4.8 minutes at pH 7.0, rises sharply to a maximum of 17.3 minutes at pH 8.2, then declines to 10.7 minutes at pH 8.6. As compared with the results of the previous experiment, the maximum on the acid side is lower and occurs at a lower pH. The neutral minimum is lower and possibly shifted slightly toward the acid side. The alkaline maximum is much increased and is shifted slightly to the alkaline side.

The curve for the percentage of deaths from coagulation starts at 45 per cent at pH 5.8, drops to about 8 per cent at pH 7.2, rises to about 28 per cent at pH 7.8, and then declines to 12 per cent at pH 8.6. The swelling was not noticeable at pH 5.8, very marked at pH 6.0, less at pH 6.8 and 7.0, more marked at pH 7.6, and again less at pH 8.6. A comparison with that in the previous experiment could not be made, as it was impracticable to place the data on a quantitative basis. The changes in the actual mechanism of death followed a course similar to that noted in the preceding experiment, i.e., coagulation in acid, bursting of the cell at neutrality, and dissolution in extreme alkalinity.

C. This experiment was carried out as before, but the KCl was omitted from the saline and replaced by molecular equivalent CaCl₂. The curves for resistance time and coagulation are presented in Figure 3.

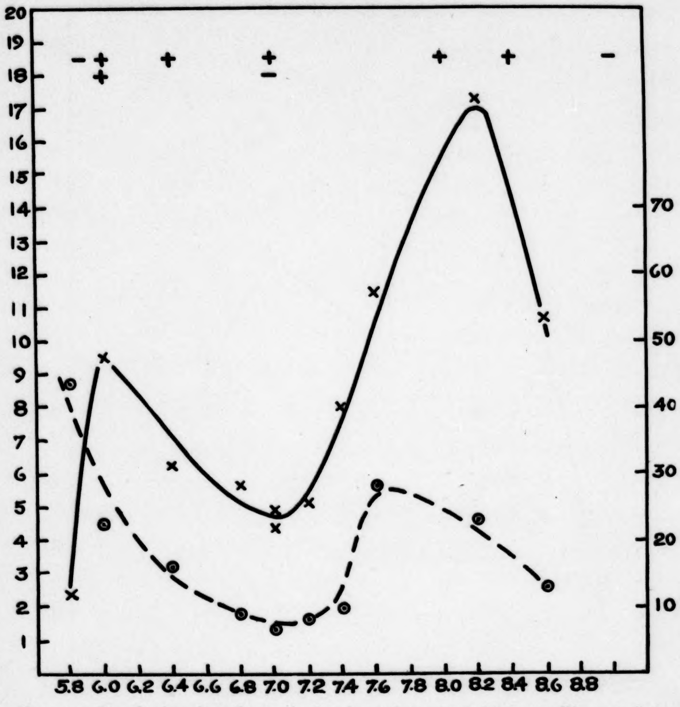


FIG. 2.—Graph showing the variation in resistance to heat, swelling, and the percentage of deaths due to coagulation in *Paramecia* exposed to 40° C. in saline with potassium excess at different hydrogen-ion concentrations. Abscissas, pH; ordinates, scale on left, time in minutes to kill 50 per cent; scale on right, percentage of deaths in which cells were unruptured and coagulated; continuous line, resistance; broken line, percentage of deaths by coagulation. Swelling indicated as follows: −none, or shrinkage; ±slight to none; +marked; $\substack{+\\+}$great.

It will be noted from these that the resistance time curve again shows two maxima; this time, however, the maxima occur at pH 6.6 on the acid side and at pH 8.0 on the alkaline side. The acid maximum is the greatest, reaching 13.5 minutes; and the alkaline is 12.5 minutes. The neutral minimum is rather wide, and apparently at pH 7.2, the resistance time here being 10 minutes. The coagulation curve drops from 52 per cent at pH 6.2 to about 2 per cent at pH 7.0,

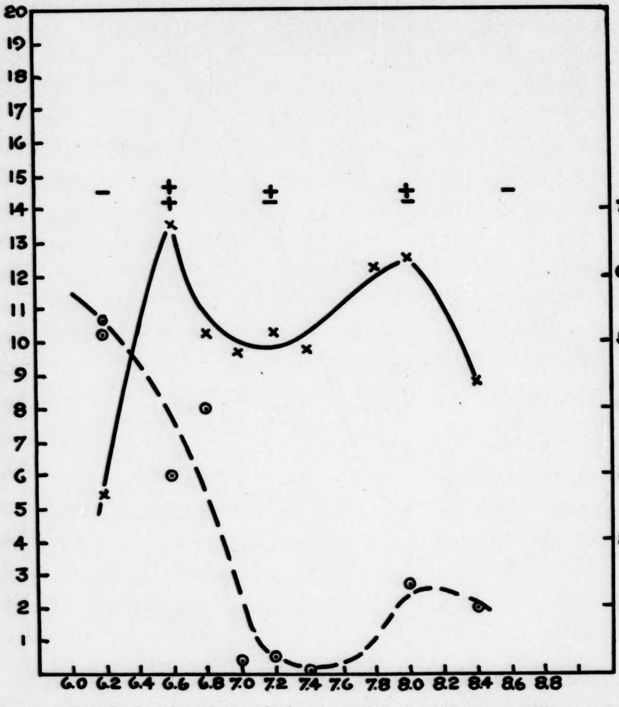


FIG. 3.—Graph showing the variation in resistance to heat, swelling, and the percentage of deaths by coagulation in *Paramecia* exposed to 40° C. in saline with calcium excess, at different hydrogen-ion concentrations. Abscissas, pH; ordinates, scale on left, time in minutes to kill 50 per cent; scale on right, percentage of deaths in which cells were unruptured and coagulated; continuous line, resistance; broken line, percentage of deaths by coagulation. Swelling is indicated as follows: −none or shrinkage; ±none to slight; +marked; ‡great.

rises to 13 per cent at Hp 8.0, and then declines to 10 per cent at pH 8.4. The swelling was, as before, negligible in extreme acidity, noticeable in the region of the acid resistance maximum, and less at neutrality. The alkaline side, however, showed *definitely* less swelling than at pH 7.0. Variation in death types was found as before, except that the degree of swelling was apparently low in the alkaline range even when coagulation increased.

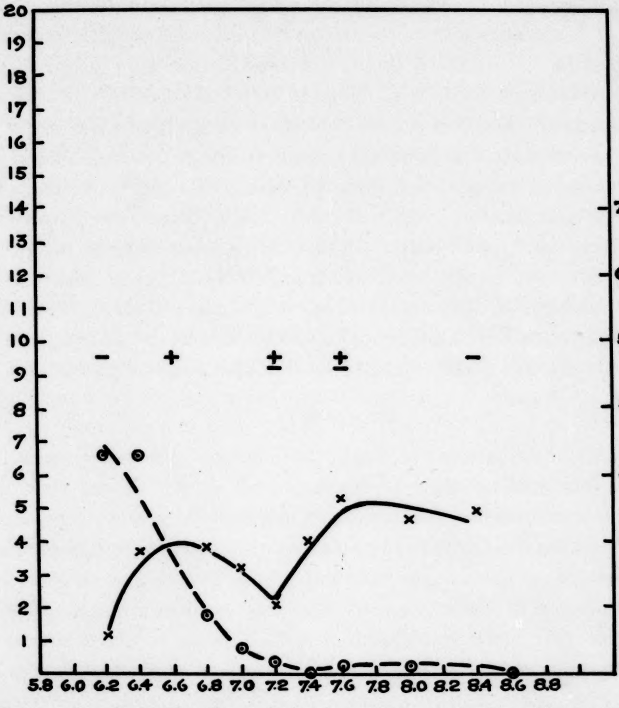


FIG. 4.—Graph showing the variation in resistance to heat, swelling, and the percentage of deaths due to coagulation in *Paramecia* exposed to 40° C. in saline with sodium excess, at different hydrogen-ion concentrations. Abscissas, pH; ordinates, scale on left, time in minutes to kill 50 per cent; scale on right, percentage of deaths in which cells were unruptured and coagulated; continuous line, resistance; broken line, percentage of deaths by coagulation. Swelling is indicated as follows: − none or shrinkage; ± none to slight; + marked; ‡ great.

D. In this experiment the same procedure was followed, but both KCl and $CaCl_2$ were omitted and sufficient NaCl was added to replace the Ca and K concentrations by Na.

The resistance time and coagulation curves obtained are given in Figure 4. From this figure it will be noted that the resistance time rises from about 1 minute at pH 6.2 to a maximum of about 4 minutes at 6.6, declines to a minimum of 2 minutes at pH 7.2, reaches a maximum of 5.2 minutes at pH 7.6, and drops very gradually to about 4.5 minutes at pH 8.4. Thus, in comparison with the previous experiments the resistance over the whole range is much lowered.

The percentage of coagulation drops from 35 per cent at pH 6.2 to 0 per cent at 7.4 and then remains at about 1 per cent or less for the rest of the alkaline range covered. The swelling noted was none at extreme acidity and markedly less throughout than in the previous experiments. It appeared also that the character of the coagulation was somewhat different. It was not so definite, and the cells appeared milky rather than densely white. It is to be noted that, as previously stated, these solutions were found to be slightly toxic.

DISCUSSION

It has been demonstrated in the preceding pages that the resistance of *Paramecium* to a temperature of 40° C. varies with the hydrogen-ion concentration, and exhibits two maxima of resistance: one in the alkaline and one in the acid range, with a region of minimal resistance at neutrality. It has also been shown that the position and magnitude of these maxima, and the minitude of the neutral minimum, vary with the inorganic salt balance in the solution in which exposure is made. Further, it has been shown that the type of death varies, death being apparently due to coagulation in extremely acid conditions, to swelling resulting in bursting of the cell membrane in the regions about neutrality, and to dissolution, with no swelling, in very alkaline solutions.

The question arises as to how these phenomena may be correlated and explained.

On considering the results obtained, it is at once obvious that since the death of the cell in the extreme acid range is apparently due to coagulation, and in the extreme alkaline range is due to dissolution, one of the factors influencing the mechanism of death is a change in aggregation of the cell colloids varying directly with hydrogen-ion concentration, and a second is a change in water content.

It appears, therefore, that the examination of the relation between these factors is the first step toward elucidation of the variations found in the mechanism of, and resistance to, the lethal process under the experimental conditions.

Now a change in aggregation must, if it affects the cell as a whole, be accompanied by an inverse change in volume. Dispersal of the cell colloids must result in swelling. It is quite evident that this relation does not hold, inasmuch as the greatest degree of swelling occurs in all solutions at the point of maximum resistance in the acid range, at which point the percentage of coagulation indicates only a moderate degree of coagulation. It is most probable, therefore, that the coagulation changes observed are not in fact due to coagulation involving the entire cell, but a part of it. The question arises as to what part is the most probable.

From examination of the curves it appears that while coagulation and swelling are not inversely related throughout, they are so related from extreme acidity to the point in the acid range at which the maximum resistance occurs. Beyond this, however, the relation is approximately direct. This is particularly noticeable in Figure 2. It seems evident, therefore, that the coagulation curves obtained indicate changes in a part of the cell closely related to the water content. It is quite obvious that this part is most probably the cell membrane, since changes in this of a coagulative nature will undoubtedly influence permeability and therefore water content.

It is concluded therefore that the curve showing the percentage of death by coagulation indicates the change in the state of aggregation of the cell membrane as affected by pH and, of course, the salts present in the experimental solutions.

Now the changes in aggregation in a membrane composed of an hydrophilic colloid passing from a condition of high coagulation to dispersal can be roughly pictured as occurring in three stages: (1) highly coagulated (the membrane colloids are in large aggregates with large interspaces; the membrane is highly permeable and probably rigid); (2) a medium state of aggregation (the colloids exist as discrete micellae, but little separated; the membrane is semipermeable to impermeable and relatively strong); (3) dispersed (the colloids exist as discrete micellae, widely separated, the membrane is permeable and fragile). Hence, in considering the curve of coagulation as indicative of the state of coagulation of the cell membrane, it represents variation in both membrane permeability and membrane strength.

While consideration of the relation between swelling and coagulation leads to an interpretation for the meaning of the coagulation changes noted, it does not provide an explanation for the cause of the swelling, since changes in permeability alone will not necessarily result in swelling. To explain swelling, a decrease in the rate of output of water from the cell, with an increase (or at least no decrease) in the rate of water intake, must be shown to occur. Because of the temperature used in the experiments, the viscosity of water is decreased below that which normally surrounds the organism; hence the intake of water will rise with increasing temperature. There is also evidence (van Herwerden, 1927; Rogers, 1927, p. 414) that at high temperatures the functioning of the contractile vacuole in Protozoa is retarded. These two conditions—increase in water intake with retarded water excretion—will result in swelling throughout the pH range where a medium state of aggregation occurs. This, for a given solution, appears to be the range between the two maxima of resistance. Now, if these factors alone are involved in swelling, it is to be expected that throughout this range in a given solution a given degree of membrane aggregation (permeability) will be accompanied, at least approximately, by a given degree of swelling. Also, since death over this range is observed to result mainly from rupture of the membrane, the time of resistance, i.e., the time necessary for the pressure to reach the limit of membrane strength, will be constant. The first requirement is apparently met, but the second is certainly not. Hence it must be assumed, since the degree of swelling before bursting is a measure of membrane strength (and this is roughly equal for equal degrees of coagulation) that the rate of swelling, upon which the time of resistance must then depend, is not the same over the pH range; i.e., it follows that the rate of water intake or the functioning of the vacuoles varies with pH. Eisenberg-

Hamburg (1929) has reported this variation in rate of water intake at lower temperatures. He finds that it is greatest at pH 7.0 and less on either side, the deceleration being most rapid on the alkaline side. If this holds at 40° C. and the function of the vacuoles is retarded, this will result in the rate of swelling in a given solution being most rapid at neutrality and less rapid in acid and alkaline solutions.

A third factor to be considered is the effect of the cations upon the cell membrane. If the coagulation curve represents the state of the membrane, the major effects may be noted directly. In the solution with excess Na or K, the main effect is to decrease coagulation in the acid range as compared with the degree present in the balanced saline, and with K but not Na to increase it in the alkaline. With this effect it would appear probable that since K or Na both penetrate protoplasm somewhat rapidly, the rate of water intake will be accelerated above that in the balanced solution because of increase in osmotic pressure. This effect corresponds to that noted by Loeb (1905, p. 511) and Gelhorn (1923) for the action of K on muscle. Calcium, on the other hand, may be expected to decrease water intake, as its usual effect is to render cells less permeable. Further, it may be noted that Gray (1926) found that in alkaline solutions Ca produced a granular precipitation and dispersal in the intracellular matrix of *Mytilis*, and this matrix he believes to be comparable to the pellicular membrane of the Protozoa.

To sum up—it can be assumed (1) that the course of the curves obtained for percentage coagulation represent changes in aggregation in the cell membrane as resulting from the ionic equilibria in the solutions used; (2) that the rate of water intake into *Paramecium* varies with pH as reported by Eisenberg-Hamburg; (3) that at 40° C. the functioning of the contractile vacuoles is retarded; (4) that excess of K or Na results in increased and excess of Ca in decreased rate of water intake, and that in alkaline solutions excess of Ca produces a granular disintegration of the cell membrane as described by Gray for the intracellular matrix in *Mytilis*.

On the basis of these assumptions the changes in resistance and types of death found will be as follows: On exposure of *Paramecium* to a temperature of 40° C. in the balanced saline, the resistance will vary with increasing pH. In extreme acidity the membrane will be strongly coagulated, and death will result from exosmosis of salts. There will be no swelling, and the cell membrane will not rupture. As the pH is increased, the coagulation will lessen, and the resistance will rise owing to slower rate of exosmosis.

With continued increase in pH this process will produce a degree of semipermeability sufficient to result in a supplanting of exosmosis by osmosis. At and beyond the point at which this change occurs death will result from rupture of the membrane, and resistance will depend upon its strength. Evidently, since the degree of swelling depends on membrane strength, and since further increase in pH will decrease this and simultaneously increase the rate of water intake, this is the point of greatest swelling and of maximum resistance in the acid range. Beyond this point increase in pH will result in decreasing membrane strength and increasing rate of water intake, and therefore in decreasing resistance until neutrality is reached. On passing into the alkaline range, coagulation and membrane strength will become approximately stationary until pH 8.0 is reached; but the rate of water intake will drop rapidly and therefore resistance will rise. The deceleration in the rate of water intake will be very great, and therefore the resistance will be much greater for a given degree of coagulation in the alkaline than in the acid range. This will probably result in a greater maximum resistance in the alkaline than in the acid range. Finally, as the pH is increased still further, the membrane will be greatly dispersed and again be permeable, with the result that resistance will decrease and death will result from dissolution of the cell with little or negligible swelling.

When exposure is made in the solution containing an excess of K, the general course of resistance will be similar to that in the balanced saline; but because of the excess of K, certain differences will obtain. The coagulation on the acid side will be less, and the water intake will be more rapid throughout. This will result in a lower maximum of resistance at low pH and a shift of this maximum toward acidity. At neutrality the coagulation will be slightly greater; but because of the increase in water intake due to K, the minimum of resistance will be lowered. On the alkaline side, as coagulation will be greatly increased, the resistance will be increased despite the increase in water intake; but because of this increase in rate, the maximum will fall at a slightly higher pH. In the solution with Ca excess coagulation in the acid range will be about as in the balanced saline; the maximum resistance will be increased owing to lessened rate of water intake, as will also the resistance at neutrality, despite the low coagulation at this point. In the alkaline range, however, this slowing of water intake will not result in an increased maximum resistance, since at this range in pH a granular dispersion of the membrane will begin. This will result in apparent coagulation increase but no correspondingly great increase in resistance or swelling.

In the saline with Na as the sole metallic cation present, the rate of water intake will be very greatly increased; and hence, despite the fact that the coagulation will be relatively high in the acid range, the maximum resistance will be low. The rise in the alkaline side will appear, but throughout the curve a low degree of resistance will obtain. Since, as pointed out, this solution is slightly toxic, it may be that the Na ion effects the membrane to reduce its strength as noted by Lepeschkin (1924, pp. 177–79).

It is of interest to note that Ephrussi and Neukomm (1927) find a bimodal curve for the resistance to heat of the eggs of the sea urchin *Paracentrotus lividus*. This has an acid optimum at about pH 6.8–7.1 and an alkaline optimum at pH 10. Whether this points to such a curve as typical for cell resistance to heat seems problematical. The mechanism producing it in the sea-urchin egg must differ from that presented here, as the imbibitional changes with pH which were also studied by these investigators are quite dissimilar from the changes in rate of water intake reported by Eisenberg-Hamburg. This is, of course, to be expected, as the absence in the sea-urchin egg of the contractile vacuoles and gullet of *Paramecium* with its resultant continuous passage of water through the cell must result in greatly different water relations.

It seems desirable to emphasize that the problem dealt with does not rest upon any concept as to the cause of heat coagulation, and it is immaterial from the standpoint taken if the initial coagulation changes are considered to result from coagulation or denaturization of proteins or the melting of fats.

SUMMARY

1. *Paramecium* exposed in saline solutions of different hydrogen-ion concentrations to a temperature of 40° C. varies in resistance to heat.

2. This variation, when expressed as a function of pH, is bimodal, with maxima of resistance in the alkaline and acid ranges and a minimum at neutrality.

3. The position and magnitude of the maxima and minitude of the minimum are dependent on the inorganic salt balance of the medium.

4. When *Paramecium* is exposed in saline solutions of different hydrogen-ion concentration to a temperature of 40° C., the mechanism of death varies. In solutions of pH 6.0 or less the cell coagulates; at pH 8.0 or more the cell disintegrates. Between these two extremes death results from rupture of the cell due to swelling.

5. A hypothesis is presented in explanation of these variations.

LITERATURE CITED

Chalkley, H. W. 1930. The resistance of *Paramecium* to heat as affected by changes in hydrogen-ion concentration and in the inorganic salt balance in the surrounding medium. U.S. Public Health Repts., XLV, 481–89.

Eisenberg-Hamburg, E. 1929. Recherches comparatives sur le fonctionnement de la vacuole pulsatile chez les infusoires parasites de la grenouille et chez les infusions d'eau douce. Influence de la pression osmotique, des electrolytes et du pH. Arch. f. Protistenkunde, LXVIII, 251–70.

Ephrussi, Boris, and Alexandre Neukomm. 1927. Résistance à la chaleur des œufs d'oursin (*Paracentrotus lividus, Lk.*). Protoplasma, II, 34–44.

Gelhorn, Ernst. 1923. Beitrag zur allgemeinen Zellphysiologie II. Weitere Studien über die Quellbarkeit quergestreifter und glatter Muskelarten, und ihre Permeabilität unter verschiedenen Bedingungen. Pflügers Arch. f. Ges. Physiol., CC, 581–603.

Gray, J. 1926. The properties of an intercellular matrix and its relation to electrolytes. Brit. J. Exp. Biol., III, 168–87.

Herwerden, M. A. van, 1927. Umkehrbare Gelatinierung durch Temperaturerhöhung bei einer Süsswasseramöbe. Protoplasma, II, 271–77.

Kahler, H.; H. W. Chalkley; and Carl Voegtlin. 1929. The nature of the effect of a high frequency electric field upon *Paramecium.* U.S. Public Health Repts., XLIV, 339–47.

Lepeschkin, W. 1924. Kolloidchemie des Protoplasmas. Berlin: Julius Springer.

Loeb, J. 1905. Studies in general physiology. Chicago: University of Chicago Press.

Rogers, Charles Gardner. 1927. Textbook of comparative physiology. New York: McGraw-Hill Co.

THE HIGH TOXICITY OF NASCENT OXYGEN[1]

CARL L. HUBBS
Museum of Zoölogy, University of [illegible]higan

I

THE experiments recounted in the present paper were begun to test the availability of certain water-purification processes for rendering an unsatisfactory water supply fit for the maintenance of delicate fishes. It was soon determined that the strong oxidizing agents, which are the basic principle of these processes, produced a very satisfactory water, provided all traces of residual oxidizing chemicals were removed. The incidental discovery that nascent oxygen is toxic to fishes and other aquatic animals, to a degree far beyond a priori expectation, led to a considerable extension of the investigation.

II

The sources of nascent oxygen used were ozone, sodium hypochlorite, and hydrogen dioxide.

Ozone was supplied in aqueous solution by an Ozonator. This machine produced ozone by a 10,000-volt arc in dried air, then injected the ozonized air into water and mixed the two gently but thoroughly in a series of concentric columns. The water delivered was almost sterile, had an extremely low oxygen demand, and was supplied with about 6 parts of ozone in 1,000,000 of water. Although most of this gas escaped on first exposure to the air, because its solubility in water under ordinary conditions is far below even that small degree, a minute though very effective residuum of ozone remained in the water.

---

[1] Acknowledgments for material equipment and for technical and scientific advice are due to the United States Ozone Company, which provided the University of Michigan with a very satisfactory Ozonator for these tests; to Dr. Frank E. Hartman, chief chemist for that company, who freely answered my questions regarding the chemistry of ozone, and to the Detroit representatives of that company and of the Marsh Chlorination Process. Colleagues at the University of Michigan contributed generously to the investigation: Frank E. Eggleton loaned tested reagents for some of the determinations, and allows reference to be made to experiments by himself on the effects of ozone on certain invertebrates; Charles E. and Paul O. Greeley assisted in the spectroscopic analyses of fish blood; Dr. Herbert W. Emerson ran the nitrite determinations and gave advice on physiological questions; and Dr. Hobart W. Willard and Floyd E. Bartell gave me the benefit of their rich chemical experience. Others helped, but to those mentioned I owe most.

In the usual experimental set-up, in which a portion of the effluent of the Ozonator was conducted through funnel and tubing into an aquarium jar, a supply of ozone was obtained which analyzed 1.2–1.8 p.p.m. when the machine was operated with 110 volts; about 0.6–0.9 p.p.m. at 95–100 volts; and a bare trace only at 85–90 volts (the variation is partly due to slight differences in the taking of the sample for analysis). The amount of ozone was regulated down toward the zero point not only by reducing the voltage but also by bubbling air through the experimental jar or through another jar intervening between it and the machine.

TABLE I

Sample Numbers	Time Taken (hrs.: min.)	Time Titrated (hrs.: min.)	Time Interval (hrs.: min.)	Cc. N/96 $Na_2S_2O_3$	P.p.m. Ozone	P.p.m. Nascent Oxygen
1	0:35	1:50	1:15	0.80	1.33	0.43
2	0:45	1:45	0:60	0.80	1.33	0.43
3	0:55	1:40	0:45	0.85	1.41	0.45
4	1:05	1:35	0:30	0.79	1.31	0.42
5	1:15	1:25	0:10	0.80	1.33	0.43

During most of the experiments, the concentration of ozone was maintained by using a running supply. That the solution remained fairly constant was indicated by a series of analyses (see Table I) of the ozone in the experimental jar, made while the Ozonator was being operated as during most of the experiments. These data also indicate that the apparatus, chemicals, and analytical methods employed permitted determinations of sufficient consistency for the present investigation, for which no high accuracy was demanded.

The procedure followed in the ozone analyses, on the advice of Dr. F. E. Hartman, was to add to the 150 cc. sample, 5 cc. of a 2 per cent solution of KI; to shake the sample at once and then again after two or more intervals of several minutes; and then to titrate very carefully with thiosulphate, using the usual starch indicator.

The amount of ozone is calculated from the amount of thiosulphate used, as each cubic centimeter of N/96 $Na_2S_2O_3$ is equivalent to 0.25 mg. of ozone, or to 0.08 mg. of available or nascent oxygen.

In the determinations of dissolved oxygen, the modified Winkler method outlined in the *Standard Methods for the Examination of Water and Sewage* was followed, with the necessary elimination of the initial potassium permanganate treatment in the samples containing available oxygen (for such samples this treatment is not needed). In the analyses of the dissolved carbon dioxide and of the nitrite, the same *Methods* were followed. In obtaining the hydrogen-ion concentration, the colorimetric method with a standard field set of indicators was employed (the ozone considerably reduced the intensity of the color of the indicator, but the quality rather than the intensity of the color was regarded as significant.)

The sodium hypochlorite solution used was obtained from a machine producing the hypochlorite electrolytically from sodium chloride, by the Marsh Chlorination Process. The concentration was computed in the earlier experiments from the rated capacity of the machine. For the latter experiments the solution, then greatly weakened by standing for several weeks, was analyzed for available oxygen by titration with N/40 $Na_2S_2O_3$ after thorough treatment with an acid solution of KI.

The hydrogen dioxide used was a 3 per cent solution, standardized by the usual method of titration with N/10 K_2MnO_4.

In the second series of hypochlorite experiments and in the dioxide tests the chemicals were diluted with water of tested non-toxicity, saturated with oxygen, and, significantly, freed from practically all readily oxidized organic matter. This water was obtained by ozonizing tap water and then completely removing the ozone by bubbling air.

A number of controls were found desirable. The method generally employed in the controls for the ozone experiments was to keep an animal, closely comparable to the one being experimented upon, in water obtained directly from the city supply, from the line leading to the Ozonator. It was necessary to aërate this water, for it comes from the tap devoid of oxygen. This was usually accomplished by shooting the water from a fine valve into a fish-hatchery jar, in which it was very effectively mixed with air, and from the overflow of which a desired amount was conducted through funnel and tubing into the control jar. The aëration in other controls was effected by compressed air, and by the use of the Ozonator when the current was turned off.

III

The symptoms of nascent-oxygen poisoning exhibited by free-swimming fishes are fairly constant, both as to character and as to sequence, and are seemingly independent of the source of the active oxygen, whether from ozone, hypochlorite, or dioxide. These symptoms have been briefly, and I believe rather imperfectly, described by Belding (1928) as the symptoms of hypochlorite poisoning, and contrasted by him with the effects of other poisons.

The initial response, usually evident as soon as the fish slows down from its first wild dash in the strange jar, is one of apparent irritation. The fish swims about actively and nervously, avoiding the bottom, and soon exhibits jerking and twitching movements, even at times a spasmodic shaking of the head.

The respiratory movements are typically increased in rate and especially in amplitude, but the fish does not ordinarily come up to lap at the surface, as it does in asphyxiation. The gill-flap movements of a mud pickerel (*Esox vermiculatus*) increased after the addition of hypochlorite from one per 2.5 seconds to one per 1.5 seconds. Especially marked and characteristic is the irregularity and spasmodicity of the respiratory movements of a fish treated with nascent oxygen. Somewhat similar respiratory changes have been described for mammals breathing ozone, and were unexpectedly shown on kimograph records made by Charles E. and Paul O. Greeley in the physiological laboratory of the University of Michigan, of the respiration of dogs into whose blood some ozonized water, made up into a normal salt solution, was injected.

These initial symptoms, of altered locomotor and respiratory movements, are displayed by fishes subjected to sublethal concentrations of nascent oxygen, certainly of less than 0.01 p.p.m. The reactions of the fish seem to be as sensitive an indicator of nascent oxygen as is the very delicate iodimetric analysis. The experiments illustrating this point are outlined later.

Before long there is to be noted a slight loss of equilibrium: a very significant symptomatic stage, in that it marks the end of the reversible effects of the poisoning. Most of the fishes which were not affected to this stage later showed an apparently complete recovery. But when this initial loss of balance is reached, death surely follows. It is impossible to save the affected fish by shutting off the poison supply, by introducing pure water or air into its container, or even by removing the fish into healthy water. Many experiments yielded the evidence on which these statements are made. One typical example may be cited: An adult minnow, *Hyborhynchus notatus*, was placed in water containing 1.5 p.p.m. of ozone; in 2.5 minutes it showed the first incipient indication of loss of equilibrium; it was then removed to normal water; there resulted, however, no recovery, but a steady and orderly procession of the poison symptoms, ending in death at 37 minutes after the transfer. Any theory proposed to explain the death-dealing property of nascent oxygen ought to harmonize with these observations.

After this initial, death-predicting loss of equilibrium, the fish always quickly recovers its balance temporarily. But before long it may be seen lying on its side for several seconds at a time. There then typically follows a very interesting symptom, the rolling and dashing stage. After passing through this, the fish tends to become quiescent for intervals between wild darts. Gradually the periods of unequilibrated rest are prolonged as the dashes become less frequent. The fish usually lies belly-up, gasping, rarely flitting from one position to another. Its color fades; tetanus sets in, and finally becomes intense; only slight gill movements remain to indicate life; the skin, especially on the under side of the head and breast, becomes more or less oedematous; then death of course ensues.

So far as their habits and structures allow, the various invertebrates experimented on by Mr. Eggleton showed on treatment with ozone a somewhat similar procession of symptoms.

These symptomatic effects of nascent oxygen poisoning are recounted in so much detail, in the hope that they will suggest to some physiologist by what action such minute quantities of nascent oxygen can kill an aquatic animal.

IV

A number of the ozone experiments with fishes may now be recounted, in the order of the concentration of nascent oxygen maintained in the experimental jar. In this way we may indicate the lethal dose and something more of the observable mode of action of this poison.

These experiments were all performed on fishes which had been caught locally in December, when their general resistance is high. All of them had been kept long enough in the laboratory to become well adjusted to aquarium conditions.

Only the conditions and results of many of the experiments can be presented. Anyone interested in the details, especially of the reactions of the fishes, may obtain fuller information from the writer.

Exp. 1.—Available nascent oxygen: bare trace (less than 0.01 p.p.m.), as the water had stood 19 hours after being ozonized.—Small adult of a minnow, *Hyborhynchus notatus:* immediately showed increased respiration and rather jerky swimming; in 5 minutes gave semispasmodic twitchings and kept to midwater; at 35 minutes appeared nearly normal and remained so until the end of the experiment (6 hours).

Exp. 2.—Available nascent oxygen: bare trace at start, remaining from operation of machine up to ½ hour previously at 111 volts, declining to no apparent trace because the machine was being operated at a low voltage (63–66).—One half-grown dace, *Rhinichthys atronasus meleagris:* showed increased respiration, slightly spasmodic twitchings, and some rather wild dashes, but no loss of equilibrium to end of experiment (more than 4 hours).

Exp. 3.—Available nascent oxygen: bare trace, immeasurable, owing to operation of machine at a reduced voltage (90) and to the bubbling of air through the experimental jar.—One half-grown each of *Hyborhynchus notatus* and *Notropis rubellus:* each exhibited at once rather jerky movements and exaggerated respiration; the *Hyborhynchus* showed once what may have been a slight loss of balance, but neither clearly lost control until end of experiment (76 minutes).

Exp. 4.—Available nascent oxygen: about 0.01 p.p.m. (reduced by air-bubbling in an intervening jar from a measured concentration of 0.02 p.p.m. at the effluent of the machine, operated at 100–105 volts).—One half-grown each of five cyprinids, *Rhinichthys atronasus meleagris*, *Semotilus a. atromaculatus*, *Notropis cornutus chrysocephalus*, *N. rubellus*, and *Hyborhynchus notatus:* all exhibited the usual symptoms of irritation; the *Notropis cornutus* showed violent twitchings and perhaps slight loss of equilibrium after more than an hour, but none showed any clear loss of control during the entire experiment, of nearly 2 hours. After this sublethal dose these fishes showed rather unusual resistance to higher concentrations of ozone (see Exp. 6 and 13).

Exp. 5.—Available nascent oxygen: 0.03 p.p.m.; reduced to this concentration from 0.85 p.p.m. by bubbling air through the intervening jar.—A small adult of *Hyborhynchus:* at once showed the usual initial symptoms, which progressed to intermittent loss of equilibrium at 16 minutes, violent rolling and darting at 20 minutes, general quiescence except for exaggerated breathing at 25 minutes; only occasional dashes and onset of tetanus at 29 minutes; extreme weakness at 39 minutes; death at 56 minutes. This fish therefore was killed in less than an hour, with all the symptoms of nascent oxygen poison, by an active oxygen concentration of less than 1 part in 30,000,000 parts of water.

Exp. 6.—Available nascent oxygen: 0.08 p.p.m.; reduced to this dilution by use of compressed air in the jar intervening between machine and experiment. —Five fishes, those which survived Experiment 4 (Experiment 4 was directly continued as Experiment 6): the *Hyborhynchus* clearly lost balance in 25 minutes, and died later, although the ozone dropped to a mere trace; the *Notropis cornutus* was affected almost as quickly, and also died later; the three other fishes survived the treatment for nearly 6 hours, although they became rather sluggish. Their survival suggests the possibility of acclimatization to nascent oxygen poison, because they had previously been subjected to a sublethal dose. Experiment 6 was directly continued as Experiment 13.

Exp. 7.—Available nascent oxygen: 0.16 p.p.m.; the machine operated at 100 volts, but the excess ozone removed by bubbling air through the experimental jar.—Another small adult of *Hyborhynchus:* within a minute exhibited the initial symptoms of nascent oxygen poisoning; at 4 minutes first lost balance and dashed wildly; at 10 minutes had lost all normal control, already showed occasional roll-and-dash movements, and would of course have soon died.

Exp. 8.—Available nascent oxygen: 0.16 p.p.m.; reduced this low by allowing the water to stand for 80 minutes.—Two adults of *Hyborhynchus:* both killed, following the usual symptoms.

Exp. 9.—Available nascent oxygen: 0.29 p.p.m.; the machine operated at 95 volts.—One large young of the *Rhinichthys:* nearly dead within 25 minutes (end of experiment).

Exp. 10.—Available nascent oxygen: about 0.4 p.p.m. at beginning of experiment, for the machine had been running on 111 volts, quickly reduced to a bare trace or none by cutting down the voltage to 65.—A large young of *Hyborhynchus:* killed in 5 hours.

Exp. 11.—Available nascent oxygen: 0.4 p.p.m. (analysis made for Case A; the concentration estimated for other cases from several analyses made under the same conditions, which include the operation of the machine at 110 volts).—*Case A*, one half-grown each of *Hyborhynchus* and of *Notropis rubellus;* both nearly dead at end of experiment (20 minutes). *Case B*, one adult of *Hyborhynchus:* put onto side, a fatal sign, in just 10 minutes (end of experiment).

Case C, one half-grown each of *Notropis cornutus chrysocephalus* and *Semotilus a. atromaculatus:* the former removed on approach to death at 70 minutes; the latter killed in about 4 hours. *Case D*, one adult each of the *Rhinichthys* and the *Hyborhynchus:* carried beyond reversible effects in 11 minutes. *Case E*, an adult chub (*Semotilus*) with a skin wound, and two dace (*Rhinichthys*): the chub lost control in the unusually short time of ½ minute; the dace reached this stage in 4 minutes; and all were lying on their sides in 10 minutes. *Case F*, a minnow (*Hyborhynchus*): died in 42 minutes.

Exp. 12.—Available nascent oxygen: higher than 0.4 p.p.m. but certainly lower than 0.8 p.p.m., the concentration in the funnel which overflowed directly into the experimental jar.—One adult each of *Hyborhynchus notatus* and of *Notropis cornutus chrysocephalus:* both showed loss of equilibrium, the death-predicting symptom, in less than 12 minutes.

Exp. 13.—Available nascent oxygen: 0.59 p.p.m.; the concentration held as high as possible with the set-up used.—Three fishes, *Rhinichthys atronasus*, *Semotilus atromaculatus*, and *Notropis cornutus*, the survivors of Experiment 6, which was directly continued into this one: the *Notropis* died in 42 minutes; the *Rhinichthys* was mostly quiescent or rolling when the machine was turned off after 60 minutes operation, and died during the night; the *Semotilus*, perhaps as a result of acclimatization, remained alive until the end of observation (18 hours), though it would probably have finally succumbed.

Many of the experiments were checked by controls, as indicated in Section II. The control fishes were kept under similar conditions, except for the lack of available or nascent oxygen. In all cases the controls lived, with no marked symptoms of distress and certainly in no case with loss of equilibrium, for the entire length of the test. In some of the controls the water used had been ozonized but then freed of ozone by bubbling air.

This degree of detail has been introduced to show (1) that all the fishes experimented on exhibited irritation symptoms, even in traces of ozone too slight to detect chemically; (2) that some minnows were unable to maintain life in an ozone solution having a concentration, in terms of nascent oxygen, of only 1 part in 33,000,000, or of 1 part in 12,500,000 or of 1 part in 6,000,000 (0.03, 0.08, and 0.16 p.p.m., respectively); (3) that different individuals of the same species showed marked variation in their susceptibility to nascent oxygen poisoning; and (4) that the greatest resistance was shown by a fish which may have become acclimated to nascent oxygen.

V

Some experiments may now be cited to indicate the effects of ozone on other aquatic animals.

Exp. 14.—Available nascent oxygen: a trace only for 55 minutes, then 0.29 p.p.m. for 95 minutes, finally 0.37 p.p.m. for 115 minutes (265 minutes in all).—One crayfish, *Cambarus immunis:* showed at first, as compared with control, a greater activity in walking, and exaggerated movements of the mouth parts and swimmerets; moved round and round the aquarium jar after the first increase in ozone concentration, but gradually became weaker; became still weaker and more sluggish after the second increase in nascent oxygen. At the end of the experiment it was transferred to aërated water with the control; but, as with the fishes under like circumstances, there was no recovery, instead, a progressive weakening leading to death in about 35 hours after the start of the experiment.

Exp. 15.—Available nascent oxygen: 0.29 p.p.m. for 65 minutes, then .37 p.p.m. for 115 minutes (3 hours in all).—One adult frog, *Rana pipiens*, free to rise to the surface to breathe air through a coarse screen: became sluggish; remained under water much longer than the control, for about 8 minutes at a time; finally became too weak and stiff to move, even on rough treatment, but had not actually died at end of experiment.

Exp. 16.—Available nascent oxygen: about 0.4 p.p.m. (1 part to 2,500,000).—Various small to minute planktonic and bottom invertebrates, including both anaërobic and aërobic types (experiments performed by Frank E. Eggleton, with the same apparatus employed in the other experiments): all more or less quickly killed.

VI

Before the observed high toxicity of the ozone treatment could be definitely accredited to its one-third content of available nascent oxygen, a number of other lethal possibilities had to be excluded.

The different behavior of the animals in the ozonized water and in the controls cannot be ascribed to any deficiency or to any supersaturation of ordinary dissolved oxygen, because in all experiments and controls the water was well charged with oxygen; and there never was an effective supersaturation of that dissolved gas because the excess was free to escape from the water, which was always disturbed in one way or another, and because the supersaturation as measured was very slight (about equivalent to the minute ozone content). The following analyses of dissolved oxygen were made.

Tap water, 0.0–0.4 p.p.m., usually no trace.
Control water, from water-aërating device described on page 443, 9.7 p.p.m.

Effluent from Ozonator when operated without electricity (not producing ozone), 10.9—11.1 p.p.m. (two tests).

Effluent of Ozonator when operated at 65 volts (producing perhaps the slightest, non-testable quantity of ozone), 10.4–11.4 p.p.m. (four tests).

Water in experimental jar, containing 1.8 p.p.m. of ozone, 12.8–13.0 p.p.m. (three tests).

Outlet of hose leading to experimental jar, under same conditions, 12.8–13.1 p.p.m. (two tests).

Overflow from funnel at outlet of ozone machine, containing 2.5 p.p.m. of ozone, 12.7–13.05 p.p.m. (two tests).

Similarly, the effects obtained can hardly be ascribed to the dissolved carbon dioxide content. The following CO_2 analyses were made.

Conditions in which the fishes lived (CO_2, 0.14–28.2 p.p.m.)

Experimental jar filled when machine was operated at 110 volts, but then aërated for 15 minutes, 0.14 p.p.m.

Control water, from water-aërator, 11.3 p.p.m.

Effluent from Ozonator, operated without electrical current, 22.2–28.2 p.p.m. (two tests).

Conditions in which the fishes died (CO_2, 0.5–27.1 p.p.m.)

Experimental jar, with aëration in jar intervening between it and machine, 0.5 p.p.m.

Overflow from funnel at outlet of Ozonator, when operated at 110 volts, 18.3 p.p.m.

Experimental jar without aëration, with machine operating on 110 volts, 24.2 p.p.m.

Tap water, not aërated, 27.1 p.p.m.

The death of the fishes in the ozonized water cannot be attributed to a change in hydrogen-ion concentration, for when the machine was given 110 volts, the pH was not decreased to a value as low as 7.2, from an original level of 7.4.

The slight change in pH also indicates that the killing agent was not the nitrate radical, although a small quantity of nitric oxide was doubtless produced in the arc.

The toxicity of the ozonized water just as surely is not due to the nitrite radical, because actual analyses (by the *Standard Methods for the Examination of Water and Sewage*) showed only a bare trace of nitrite. The exact amount was not actually determined but was certainly far below any lethal dose, and apparently well below

0.01 p.p.m., for a standard prepared from tap water, with nitrite added to the amount of 0.01 p.p.m., showed a stronger color in the colorimetric test.

The lethal cause was not the trace of copper or of other heavy metals which might have been dissolved by the ozone in passing through the mixing columns. No such compounds would be eliminated or broken down by merely bubbling air through the poisonous water for 2½ minutes, which is long enough to destroy all its toxicity.

Dr. Floyd E. Bartell, who has carried on extensive investigations into the production of cyanides by electrical and other means, expressed confidence that none of these violent poisons would be produced in the Ozonator.

Temperature changes in the experiments and controls were insignificant: when, as usual, running water was used, the deviation did not exceed 1° on either side of 10.0° C.; when the water was allowed to stand, it never rose as high as 17°, and usually not nearly so high; and the change was never rapid, for the room was kept cool.

Having thus eliminated all other logical possibilities as to the killing principle in the ozone experiments, I concluded that the nascent oxygen was the cause. This conclusion was strongly confirmed, when I determined that hypochlorites and hydrogen dioxide, which agree chemically with ozone only in that each contains 1 atom per molecule of available oxygen, are also highly toxic to fishes, and that these substances, in killing fishes, produce the same chain of peculiar symptoms[1] which are exhibited in ozone poisoning.

VII

In two series of experiments, the toxicity of sodium hypochlorite to fishes was determined. The hypochlorites are well known to be unstable, reacting with water, probably through the instantaneous release of chlorine, to form nascent oxygen and chlorides. The rate of reaction depends on the amount of oxidizable material present.

As already mentioned, the supply of sodium hypochlorite used in both series of experiments was a concentrate obtained from a machine operating the Marsh Chlorination Process. For the first series (Exp. 17–19) the concentration was estimated from the rating of the machine; for the second series, by analysis.

---

[1] The symptoms of hypochlorite poisoning briefly outlined by Belding (1928) are at partial variance with our observations.

The high toxicity of calcium hypochlorite is indicated by its frequent use as a poison in collecting fishes. The toxicity of calcium hypochlorite to fishes has been roughly determined by several investigators, whose work is summarized by Suter and Moore (1922) and by Belding (1928): concentrations as low as 0.5 p.p.m., in terms of the whole calcium hypochlorite molecule, have been reported fatal to trout by Weigelt. Its effectiveness as a germicide is well known.

Exp. 17.—Available nascent oxygen: 0.5 p.p.m.—The first hypochlorite experiment was performed in a large, shallow aquarium, containing in its 16 cubic feet of water six adults of the mud pickerel, *Esox vermiculatus*, and several half-grown bass, *Aplites salmoides*, all thoroughly acclimated to the tank. There were also present many fresh-water mussels and some coarse organic material, all of which must have consumed a considerable part of the hypochlorite. The concentrated solution was carefully mixed into the water to yield the required dilution. Strong initial symptoms of nascent-oxygen poisoning, especially increased rate and amplitude of respiratory movements, was evident for all the fishes. The dosage was insufficient to kill any within an hour, probably because most of the nascent oxygen was consumed by the organic matter. At the end of the hour, the fishes had regained apparent normalcy, and the "chlorine odor" had about disappeared. It was assumed that the chemical was almost completely broken down.

Exp. 18.—Available nascent oxygen: 1.0 p.p.m.—This experiment was a direct continuation of the last and was carried out in the same manner and in the same tank. The poison symptoms which appeared at once in the pickerel and steadily progressed were the same as those produced by ozone: increased respiration, violent twitching, sporadic dashing, rolling, general quiescence, death. At 1 hour, just after one pickerel had died, fresh water and compressed air were turned on; but, as in the ozone experiments, to no avail, for the others died later (one survived until the next day). None of the more resistant bass reached the stage of loss of equilibrium.

Exp. 19.—Available nascent oxygen: 2.0 p.p.m.; the solution made up in a balanced aquarium to which the former occupants were returned at intervals.—Four species of fish were used, one adult chub, *Nocomis micropogon;* one adult shiner, *Notropis cornutus chrysocephalus;* one young and one yearling sucker, *Hypentelium nigricans;* and one adult top minnow, *Fundulus notatus*. The fishes were all killed, with the exception of the *Fundulus*, which was added after the "chlorine odor" had about vanished. But a shiner added even later, when the available nascent oxygen must have been very much below 1.0 p.p.m., was slowly killed. All the fishes passed through the usual symptoms of nascent-oxygen poisoning.

In the second series of hypochlorite experiments, better controlled conditions obtained. The sodium-hypochlorite solution, weakened by standing, now contained 73 p.p.m. of available oxygen (average of two titrations). This was mixed for each experiment with 5 liters of water of tested non-toxicity and of minimum oxygen demand, produced by ozonizing water and then removing the residual ozone by air-bubbling.

As controls for the second series of experiments, the four fishes later used in Experiment 24 were kept in normal condition for 79 minutes under identical conditions, except that not any of the hypochlorite solution was added to the deozonized water. In another control, four adults of *Hyborhynchus notatus* were maintained in normal condition for 48 hours in water identically treated.

Exp. 20.—Available nascent oxygen: 0.007 p.p.m.—Two cyprinids, *Hyborhynchus notatus* and *Notropis cornutus chrysocephalus:* both maintained in normal condition for 1½ hours.

Exp. 21.—Available nascent oxygen: 0.015 p.p.m.—One each of same two species and of *Rhinichthys atronasus meleagris:* all showed some initial symptoms, including twitchings, but all were nearly normal at end of experiment (3 hours). The *Notropis* died during the night in aërated water but probably not from the poisoning.

Exp. 22.—Available nascent oxygen: 0.07 p.p.m.—One half-grown shiner, *Notropis rubellus,* and one fingerling bass, *Micropterus dolomieu:* both showed the initial irritation; the shiner passed through the usual symptoms up to rolling at 16 minutes but was slow in dying (more than 3 hours); the more resistant bass did not lose equilibrium but was greatly weakened.

Exp. 23.—Available nascent oxygen: somewhat less than 0.7 p.p.m.; some removed by air-bubbling for 5 minutes.—One adult *Hyborhynchus:* killed.

Exp. 24.—Available nascent oxygen: somewhat less than 0.7 p.p.m.; reduced by standing 79 minutes, during which two fishes were killed.—One half-grown each of *Rhinichthys, Notropis* (*rubellus*), *Hyborhynchus,* and one fingerling of *Micropterus dolomieu:* the minnows all killed, with usual symptoms; the bass only weakened.

Exp. 25.—Available nascent oxygen: 0.7 p.p.m.—One half-grown each of *Notropis cornutus chrysocephalus* and of *Hyborhynchus:* killed in 76 and 61 minutes, respectively.

Exp. 26.—Available nascent oxygen: somewhat less than 7.0 p.p.m. (reduced by 5 minutes of violent air-bubbling).—One half-grown of the same *Notropis:* killed in 31 minutes.

The history of these experiments is abstracted and presented to show how alike and uniform are the reactions of fishes to hypochlorite and to ozone. Since these two chemicals agree only in possessing 1 atom of available oxygen, it is logical to assign their very similar toxic properties to nascent oxygen. The effects of the hypochlorite, however, are often assigned by sanitary engineers, to "free chlorine" or to toxic organic chlorine compounds produced as a side product of the decomposition of hypochlorite in the presence of organic matter. The existence and action of free chlorine as such, except in the oxidation reactions, is probably mythical. The possibility of the production of noxious chlorine compounds is not excluded in my first series of hypochlorite experiments (17–19) but is virtually removed from consideration in the second series (Exp. 20–26), because these were performed with water almost completely freed of organic matter by the ozone treatment.

Dissolved chlorine probably also acts as a poison through the production of nascent oxygen ($H_2O + 2\ Cl = 2\ HCl + O$). The toxicity of dissolved chlorine to fishes approaches that of ozone: Taylor and James (1928) killed trout in a concentration of 0.3 p.p.m. The symptoms recorded are those of nascent oxygen poisoning: "a moderate restlessness, with slight dyspnoea (rapid breathing) and loss of equilibrium, followed by death in two hours or less." Incidentally we may remark that the "chlorine odor" attributed to ozone is probably due to the action of chlorine on the olfactory sense organs through the formation of ozone.

The assumption that the toxicity of both ozone and hypochlorite is due to nascent oxygen finds a confirmation in the similar effect of another compound which liberates free oxygen, namely, hydrogen dioxide.

VIII

The hydrogen-dioxide experiments closely followed the procedure used in the second series of hypochlorite tests, and the controls for those tests are therefore applicable to the present experiments.

In the case of the dioxide, one special control was required, because the "3 per cent hydrogen peroxide" used was stabilized with acetanilid, itself not free of toxicity. Solutions were made up of that chemical, with deozonized water, to a concentration as great as that of the two strongest solutions of peroxide used (Exp. 32 and 33). In the lower concentration two adults of *Rhinichthys atronasus*, and in the higher concentration, another of that species and one of *Hyborhynchus notatus*, all lived without marked changes through the 100 minutes of the control experiment.

Five concentrations were employed, obtained by mixing 1, 5, 10, 50, and 100 cc. of 3 per cent H_2O_2, standardized against K_2MnO_4, with 5 liters of ozonized and then deozonized water.

Exp. 27.—Available nascent oxygen: 2.8 p.p.m.—One adult minnow, *Hyborhynchus notatus*, and one fingerling bass, *Micropterus dolomieu:* the former showed only irritation symptoms for 6½ hours, but died later; the bass, as in the hypochlorite experiments, grew weak but lived through.

Exp. 28.—Available nascent oxygen: 14 p.p.m.—An adult each of *Hyborhynchus* and *Rhinichthys:* both slowly developed the usual irritation symptoms, but both were eventually killed.

Exp. 29.—Available nascent oxygen: somewhat less than 28 p.p.m.; reduced by air-bubbling for 5 minutes and standing another 5 minutes.—One adult of *Hyborhynchus* killed.

Exp. 30.—Available nascent oxygen: 28 p.p.m.—One adult each of *Hyborhynchus* and of *Cottus b. bairdii:* both showed the irritation symptoms, which progressed in 2.7 hours to death for the minnow but only to weakness in the sculpin.

Exp. 31.—Available nascent oxygen: probably low, but shown to be present by qualitative test; conditions as for next experiment except that air was violently bubbled through the solution for 3 days.—One adult *Hyborhynchus:* killed in 3 hours.

Exp. 32.—Available nascent oxygen: 138 p.p.m.—One adult *Hyborhynchus* and one half-grown of *Notropis rubellus:* both killed, following the typical symptoms of nascent-oxygen poisoning already recounted.

Exp. 33.—Available nascent oxygen: 276 p.p.m.—Two adults of *Hyborhynchus notatus:* both died, following typical symptoms, but not until after ½ hour (in less than 2 hours).

It is clear from these experiments that hydrogen dioxide is also powerfully toxic to cyprinid fishes. It induces a series of peculiar poison symptoms identical with those produced by ozone and by hypochlorite. These observations validate the theory that the violent toxic principle in all three experiments is none other than nascent oxygen.

It is also clear that H_2O_2 is much less quickly toxic than the two other poisons, when the concentrations are expressed in the common term of available free oxygen. This is doubtless related to the greater stability of the dioxide. A highly toxic dose of ozone can be completely removed by bubbling air through the solution for only 2.5 minutes: all but a minute non-toxic amount of hypochlorite was destroyed by 3 days' air-bubbling, but an acutely toxic amount of H_2O_2 remained after such treatment.

IX

Another set of experiments was carried out in order to estimate the absolute quantity of nascent oxygen lethal to the minnow *Hyborhynchus*. Four 6.5-gram adults were used, each in 0.5 liter of ozonized water, in a small battery jar.

The first subject was used as a control. It remained alive and apparently normal for more than 2 hours, except for somewhat heavy respiration, in 5 liters of previously aërated but unozonized tap water.

Exp. 34.—The second fish was put into 0.5 liter of water ozonized at 110 volts, and containing 0.48 p.p.m. of available oxygen (average of two tests, of 0.46 and 0.50). It was therefore subjected to 0.24 mg. of available nascent oxygen, for 2.5 minutes, when it showed the first incipient loss of equilibrium (from which, as usual, it could not be resuscitated in healthy water). Immediately on removal of the fish, the water was again analyzed, and was found to contain a residuum of 0.02 mg. of available free oxygen. The amount of oxygen lost during the fatal 2.5 minutes was 0.22 mg. The amount lost without action on the fish was roughly determined as 0.08 mg., the measured loss in a similar jar containing no fish. The amount of nascent oxygen acting in any way on the 6.5-gram fish was therefore 0.14 mg., or 0.00002 part of the weight of the fish.

Exp. 35.—The third 6.5 gram-fish was put through the same procedure except that it was not removed until its death at 53 minutes. The water then contained only a bare trace of ozone. Assuming the same loss of 0.08 mg. without action on the fish, the toxic dose was somewhat less than 0.00003 part of the weight of the fish.

Exp. 36.—The last 6.5-gram *Hyborhynchus* was treated similarly except that the solution was weakened by 35 seconds of air-bubbling to a concentration of 0.35 p.p.m. of available nascent oxygen (average of two tests, of 0.34 and 0.36). It was therefore subjected to 0.17 mg. of available oxygen, instead of the fatal 0.24 mg. It showed some symptoms of distress but soon became almost normal, remaining so throughout the 113 minutes of the experiment. Assuming that about 0.05 mg. of available oxygen was in this case lost without action on the fish, then this *Hyborhynchus* survived the action on it of nearly 0.00002 part of its own weight.

These experiments indicate that adult minnows (*Hyborhynchus*) may be killed by the action on them of two or three hundred-thousandths part of their own weight of nascent oxygen but may survive treatment with nearly 0.00002 part of their weight. When the total supply of the poison solution was limited to 5 liters, the fish resisted a concentration of 0.35 p.p.m. (Exp. 36), but when the total supply was not so limited, another example of the same species was killed in less than an hour in a concentration of only 0.03 p.p.m. (Exp. 5). It is concluded that the effect of nascent oxygen is a cumulative one. The toxicity of nascent oxygen is extremely high only when the concentration is maintained at the low lethal level.

X

A plausible suggestion as to the physiological effect of nascent oxygen on fishes was strengthened by the actual statement in certain textbooks[1] that ozone, like some other poisons, transforms the haemoglobin of the blood into methaemoglobin, which, because of its stability, fails to serve the respiratory function. But this suggestion and statement proved to be erroneous, at least as applied to fishes. The blood of minnows killed by ozone, just like the blood of the controls, showed the bright-red color of oxyhaemoglobin rather than the brown of methaemoglobin, and gave the characteristic spectrum of oxyhaemoglobin. Even bubbling ozonized air through fish blood, and then diluting it with ozonized water, failed to alter the respiratory pigment into methaemoglobin. That none of the haemoglobin was so altered became evident from a collateral experiment. The haemoglobin in fish blood was readily changed to methaemoglobin by nitrite treatment. Only a trace of this altered blood, when mixed with normal fish blood, gave even with extreme dilutions of the mixture, the characteristic spectrum of methaemoglobin.

The fact that many invertebrates devoid of haemoglobin succumbed readily to minute quantities of nascent oxygen also indicates that the toxic effect is not on the respiratory pigments.

Investigations of the effect of ozone on mammals has also indicated that the nascent oxygen does not act directly on the blood. It has been found that no trace of ozone is exhaled, even though a lethal other poisons, when the concentrations are expressed in the common term of available free oxygen. This is doubtless related to the greater stability of the dioxide. A highly toxic dose of ozone can be completely removed by bubbling air through the solution for only 2.5 minutes: all but a minute non-toxic amount of hypochlorite was destroyed by 3 days' air-bubbling, but an acutely toxic amount of H_2O_2 remained after such treatment.

---

[1] See, for instance, Bodansky (1927, p. 159).

IX

Another set of experiments was carried out in order to estimate the absolute quantity of nascent oxygen lethal to the minnow *Hyborhynchus*. Four 6.5-gram adults were used, each in 0.5 liter of ozonized water, in a small battery jar.

The first subject was used as a control. It remained alive and apparently normal for more than 2 hours, except for somewhat heavy respiration, in 5 liters of previously aërated but unozonized tap water.

Exp. 34.—The second fish was put into 0.5 liter of water ozonized at 110 volts, and containing 0.48 p.p.m. of available oxygen (average of two tests, of 0.46 and 0.50). It was therefore subjected to 0.24 mg. of available nascent oxygen, for 2.5 minutes, when it showed the first incipient loss of equilibrium (from which, as usual, it could not be resuscitated in healthy water). Immediately on removal of the fish, the water was again analyzed, and was found to contain a residuum of 0.02 mg. of available free oxygen. The amount of oxygen lost during the fatal 2.5 minutes was 0.22 mg. The amount lost without action on the fish was roughly determined as 0.08 mg., the measured loss in a similar jar containing no fish. The amount of nascent oxygen acting in any way on the 6.5-gram fish was therefore 0.14 mg., or 0.00002 part of the weight of the fish.

Exp. 35.—The third 6.5 gram-fish was put through the same procedure except that it was not removed until its death at 53 minutes. The water then contained only a bare trace of ozone. Assuming the same loss of 0.08 mg. without action on the fish, the toxic dose was somewhat less than 0.00003 part of the weight of the fish.

Exp. 36.—The last 6.5-gram *Hyborhynchus* was treated similarly except that the solution was weakened by 35 seconds of air-bubbling to a concentration of 0.35 p.p.m. of available nascent oxygen (average of two tests, of 0.34 and 0.36). It was therefore subjected to 0.17 mg. of available oxygen, instead of the fatal 0.24 mg. It showed some symptoms of distress but soon became almost normal, remaining so throughout the 113 minutes of the experiment. Assuming that about 0.05 mg. of available oxygen was in this case lost without action on the fish, then this *Hyborhynchus* survived the action on it of nearly 0.00002 part of its own weight.

These experiments indicate that adult minnows (*Hyborhynchus*) may be killed by the action on them of two or three hundred-thousandths part of their own weight of nascent oxygen but may survive treatment with nearly 0.00002 part of their weight. When the total supply of the poison solution was limited to 5 liters, the fish resisted a concentration of 0.35 p.p.m. (Exp. 36), but when the total supply was not so limited, another example of the same species was killed in less than an hour in a concentration of only 0.03 p.p.m. (Exp. 5). It is concluded that the effect of nascent oxygen is a cumulative one. The toxicity of nascent oxygen is extremely high only when the concentration is maintained at the low lethal level.

X

A plausible suggestion as to the physiological effect of nascent oxygen on fishes was strengthened by the actual statement in certain textbooks[1] that ozone, like some other poisons, transforms the haemoglobin of the blood into methaemoglobin, which, because of its stability, fails to serve the respiratory function. But this suggestion and statement proved to be erroneous, at least as applied to fishes. The blood of minnows killed by ozone, just like the blood of the controls, showed the bright-red color of oxyhaemoglobin rather than the brown of methaemoglobin, and gave the characteristic spectrum of oxyhaemoglobin. Even bubbling ozonized air through fish blood, and then diluting it with ozonized water, failed to alter the respiratory pigment into methaemoglobin. That none of the haemoglobin was so altered became evident from a collateral experiment. The haemoglobin in fish blood was readily changed to methaemoglobin by nitrite treatment. Only a trace of this altered blood, when mixed with normal fish blood, gave even with extreme dilutions of the mixture, the characteristic spectrum of methaemoglobin.

The fact that many invertebrates devoid of haemoglobin succumbed readily to minute quantities of nascent oxygen also indicates that the toxic effect is not on the respiratory pigments.

Investigations of the effect of ozone on mammals has also indicated that the nascent oxygen does not act directly on the blood. It has been found that no trace of ozone is exhaled, even though a lethal dose is being inhaled. The work on mammals has been thus summarized by Cushny (1924, p. 582):

---

[1] See, for instance, Bodansky (1927, p. 159).

> Ozone . . . . is rapidly decomposed by living matter, and is certainly not absorbed into the blood unchanged; in fact it is immediately destroyed in the pulmonary surfaces. In man its inhalation in a dilution of 2–3 per million of air causes drowsiness and headache. . . . . In animals 15–20 parts of ozone per million of air sometimes proves fatal in a few hours from respiratory irritation; a condition of weakness and drowsiness precedes death, apparently as a result of local irritation, and the lessened movement is accompanied by a fall in the CO_2 eliminated.

The effect of ozone on experimental mammals has been explained, especially by Jordan and Carlson (1913), as due to a modification of the respiratory membranes or to the stimulation of the nerve-endings. The effect is probably not on the respiratory pigments. Death is presumably not due to a lessened respiratory exchange induced by an injury of the respiratory surfaces, for the blood of fishes killed by nascent oxygen is bright red and gives the spectrum of oxyhaemoglobin rather than that of reduced haemoglobin; furthermore, the fishes, in dying, did not show the symptoms of asphyxiation.

Any true explanation of the killing effect of nascent oxygen should harmonize with the observations which are recorded in this paper, and which may be thus summarized:

1. Minnows may be killed by available nascent oxygen in the dilution of 1 part to more than 30,000,000 of water.

2. Minnows may be killed by only slightly more than 2 units of nascent oxygen to 100,000 parts of their own weight.

3. The effect of nascent oxygen is cumulative.

4. The effect (on minnows) is irreversible beyond the stage of first loss of equilibrium but is generally reversible until that stage is reached.

5. The least stable nascent-oxygen-yielding chemical tested (ozone) is toxic in much lower concentrations than the most stable one (H_2O_2).

6. Different individuals of the same species show marked variation in their susceptibility to this poison.

7. Some fishes, as the basses and sculpin (*Micropterus*, *Aplites*, and *Cottus*) are much more resistant than the minnows (Cyprinidae).

8. The effect of the nascent oxygen is apparently not on the blood pigments.

9. Fishes are irritated by dilutions of ozone too weak to detect iodimetrically (doubtless less than 0.01 p.p.m.).

10. The symptoms of the poisoning are not those of asphyxiation: there is no lapping at the surface.

11. The effects of nascent oxygen, irrespective of its chemical source, are, in turn, irritation, increased and irregular respiration, loss of equilibrium, wild rolling and dashing, tetanus, death.

12. Death is accompanied by an oedematous swelling of the skin.

Pertinent observations of others are that small quantities of ozone in the air are fatal to mammals, and, I have been told, even to large plants; that ozone is toxic to a wide range of small invertebrates, both aërobes and anaërobes; that active oxydizing agents are very effective germicides. The fatal effect of oxygen on protozoan parasites and their hosts (Cleveland, 1925) and on insect larvae (Matheson, 1930) may be due to the presence of traces of ozone in the ordinary oxygen used.

Obviously, some function of living tissue is destroyed by nascent oxygen. The effect on the micro-organisms may be direct, but the killing of large organisms must follow from some superficial effect, because ozone cannot maintain itself in the presence of organic matter. It is extremely doubtful that the nascent oxygen can penetrate unchanged through the respiratory membranes into the blood. It may also be questioned whether it can diffuse through the several tissue layers separating the nerve-endings from the exterior. I hypothecate that the killing of the larger organisms is more probably an indirect result of some chemical alteration of the superficial cell layer than of the mere stimulation of the nerve-endings.

With this untested hypothesis, I must now content myself to present my results as they stand to physiologists, in the hope that they will carry on the investigation of this fascinating problem. Fishery and aquarium officials will find some points of interest in the usefulness of strong oxidizing agents in the purification of unsatisfactory waters for fish cultural and aquarium purposes, and in the danger of any residual available nascent oxygen. And perhaps pharmacologists and sanitary engineers may be interested in the demonstration of the very high toxicity of nascent oxygen, because this is the active principle of ozone, peroxide, chlorine, and hypochlorites, all used widely in the treatment of wounds and disease and in the purification of drinking water and swimming water.

LITERATURE CITED

BELDING, DAVID L. 1928. Toxicity experiments with fish in reference to trade waste pollution. Trans. Amer. Fish. Soc., 1927, pp. 100–19.

BODANSKY, MEYER. 1927. Introduction to physiological chemistry. New York: John Wiley & Sons.

CLEVELAND, L. R. 1925. The effects of oxygenation and starvation on the symbiosis between the termite *Termopsis* and its intestinal flagellates. Biol. Bull., XLVIII, 309–26.

CUSHNY, ARTHER R. 1924. A text book of pharmacology and therapeutics. 8th ed. Philadelphia and New York: Lee & Febinger. 706 pp.

JORDAN, EDWIN O., and A. J. CARLSON. 1913. Ozone: its bactericidal, physiologic and deodorizing action. Jour. Amer. Med. Assoc., LXI, 1007–12.

MATHESON, ROBERT. 1930. The utilization of aquatic plants as aids in mosquito control. Amer. Nat., LXIV, 56–86. 12 figs.

SUTER, RUSSELL, and EMMELINE MOORE. 1922. Stream pollution studies. New York Conservation Commission. 25 pp.

TAYLOR, R. S., and MILTON C. JAMES. 1928. Treatment for removal of chlorine from city water for use in aquaria. App. 7 to Rep. U.S. Comm. Fish., 1928, pp. 322–27.

EFFECT OF EXPERIMENTAL HYPERTHYROIDISM ON REPRODUCTIVE PROCESSES OF FEMALE ALBINO RATS

CHARLES K. WEICHERT
Department of Zoölogy, University of Cincinnati

THAT some relationship exists between the thyroid gland and reproductive organs seems fairly well established. Occasional statements are found in the literature indicating such a relationship, but no specific mechanism involved has as yet been demonstrated. Hoskins (1910) fed thyroid to pregnant guinea pigs in order to determine whether such feeding influenced the glandular weight of the offspring. He observed that abortions were frequent in his treated animals. Gudernatsch (1915*a*, 1915*b*) showed that feeding albino rats with fresh thyroid gland in relatively large doses resulted in emaciation, diarrhea, muscular weakness, and finally death. The hair became yellowish, stood erect, and sometimes fell out in patches. Regulation of the dose so as to keep the animals in apparently good health resulted in the fur becoming shabby and the failure of the animals to breed. Pregnancy did not occur until several weeks after feeding of the thyroid had been discontinued. If pregnancy finally did occur, the young were aborted or died soon after birth. In later-occurring pregnancies the young lived, but their growth-rate was not so rapid as that of normal animals. Kunde, Carlson, and Proud (1929) subjected rabbits to thyroid feeding experiments. The processes of oestrum, ovulation, fertilization, migration of ova, and implantation took place; but in most cases the young were resorbed during the latter two-thirds of pregnancy. Chidester and Insko (1929) fed rats on various diets in some of which desiccated thyroid was included. Pregnancies appeared in all lots. Thyroid dosage induced injury to the young during lactation but did not inhibit pregnancy or cause resorption of the young. Some of the rats died while littering.

Preliminary to a study of the relationship of the thyroid gland and the reproductive system, a series of experiments was carried out in which desiccated thyroid was fed to pregnant and non-pregnant albino rats of Wistar Institute stock. The glandular material used was the preparation put on the market by the Parke Davis Company. In order to insure the consumption of a definite amount of the thyroid material, the powder was weighed and mixed with enough water to make a thin paste. This was taken up in a pipette and fed directly to the rat. Only those animals were used that had shown at least five regular oestrous cycles before the experiments were begun.

PREGNANT RATS FED 0.25 GRAM OF DESICCATED THYROID DAILY

Ten mature female rats were placed with males and daily vaginal smears taken. If no nucleated or cornified cells were found in the smear by the fifth day after the first oestrum occurred in the breeding-cage, it was taken for granted that the rats were pregnant, and daily feeding of 0.25 gram thyroid to each rat was begun. The condition of pregnancy was later confirmed by palpation and by the appearance of blood in the vagina, as described by Long and Evans (1922). Two of these animals resorbed their young; and three died, one on the fourteenth day and two on the twentieth day. Death presumably was caused by the toxic products formed during the process of resorption or else by extensive hemorrhage during the attempt to deliver. The five remaining animals delivered young on the twenty-third to the twenty-fifth days, and one died 2 days after littering. The young were born dead or died within 1 or 2 days. In most cases the mothers made no attempt to care for their offspring. Many were partially or entirely eaten, although cannibalism of this sort appeared very infrequently in the colony. It is not uncommon, however, for normal rats to eat their offspring when born dead or when they die during the first few days after birth.

PREGNANT RATS FED 0.5 GRAM OF DESICCATED THYROID DAILY

In nine other rats the same procedure was followed, but the amount of thyroid fed daily was increased to 0.5 gram. Two of these animals resorbed their young early in pregnancy, for palpation did not reveal the presence of fetuses although the smear history and the appearance of blood in the vagina indicated that these animals were pregnant. Four rats died between the twenty-first and twenty-fourth days, either just before or during delivery. One of the remaining three delivered dead fetuses on the twenty-first day, the other two delivering dead fetuses on the twenty-fourth day. One of these two rats was killed to provide material for histological study, and the other died 6 days after delivery. The animal which delivered on the twenty-first day recovered. The delivered young in this experiment were, in several cases, found with the placentas still attached.

All of the rats undergoing the experimental treatment appeared to be in excellent health until the last 2 or 3 days of pregnancy, when they appeared very much weakened, perhaps from the loss of blood during the attempt to deliver.

NORMAL RATS FED 0.25 GRAM OF DESICCATED THYROID DAILY

In order to determine whether thyroid feeding has any effect on the oestrous cycle, twelve normal rats which had shown regular oestrous cycles were fed 0.25 gram of desiccated thyroid daily. These animals had, on an average, 2.1 regular cycles, after which the vaginal smears presented the typical dioestrous picture, only leucocytes being present. The dioestrous condition persisted as long as thyroid feeding was continued. Five of the rats were killed to provide material for histological investigations. Feeding thyroid to the remaining seven was discontinued after the dioestrous condition had persisted for a period of from 6 to 22 days. All of these animals came into oestrus 2–10 days after feeding was discontinued, the average being 6 days.

One rat, C20, which is not included in the foregoing, was an exception. This rat had two oestrous cycles after feeding was begun, and then the vaginal smear showed only leucocytes for 15 days. On the sixteenth day the animal came into heat despite the fact that thyroid feeding was continued. After this the dioestrous condition returned and persisted for 31 more days, the animal coming into oestrum on the ninth day after feeding was discontinued.

The general health and appearance of these rats were not markedly altered. The hair coat became somewhat rougher, and diarrhea occurred in some cases. After 3 weeks of thyroid feeding, as was determined in five animals, the body weight had decreased approximately 19 per cent. The normal weight was reattained about 2–3 weeks after feeding was stopped.

DISCUSSION

These results indicate that experimental hyperthyroidism has a very direct effect on the reproductive processes of both normal and pregnant rats. Serial sections of the ovaries of two hyperthyroid rats were made. One had been fed thyroid for 18 days and had been in dioestrus for 10 days. The other had been fed thyroid for 30 days and had been in dioestrus for 23 days. These sections very much resembled those of normal ovaries in that a number of corpora lutea, some rather large in size and some undergoing involution, were found along with follicles of all sizes. It seems that all of the corpora lutea should have involuted by the twenty-third day unless some influence had been exerted on them which caused them to persist. Not enough histological data have as yet been obtained to warrant the formation of any definite conclusions as to the mechanism of oestrus inhibition by experimental hyperthyroidism. It seems possible, however, that the hyperthyroid condition stimulates the production of the lutealizing hormone by the anterior lobe of the pituitary body. This, then, may act upon the ovary, inhibiting oestrus by causing the corpora lutea to persist. The fact that many corpora lutea are found in the ovary after a long dioestrus induced by thyroid feeding seems to bear this out. The physiological processes involved are being further investigated.

The observations of Gudernatsch (1915*a*, 1915*b*), whose treated rats would not breed, can be explained in the light of the foregoing; i.e., the dioestrous condition persisted as long as the animal was under the hyperthyroid influence, and therefore the animals would not breed. Chidester and Insko (1929) probably would have secured some cases of resorption and of inhibition of pregnancy had they fed larger doses of thyroid.

One noticeable effect of experimental hyperthyroidism in the pregnant rat is the prolongation of the gestation period in those rats which lived long enough to deliver their young. Observations made by Long and Evans (1922) show that in normal rats 90 per cent of the gestation periods fall between 21.5 and 22 days. In but one of the eight experimental rats which delivered young was the gestation period as short as this. This rat delivered on the twenty-first day. Of the others, one delivered on the twenty-third day, three on the twenty-fourth day, and three on the twenty-fifth day. From superficial observation the prolongation of the gestation period seems to be due to the inability to deliver rather than to the failure of the animals to come to full term at the normal time. It is of interest to note in this connection that Teel (1926) obtained a similar reaction in rats injected with a sodium-hydroxide extract of the anterior lobe of the pituitary body.

Two rats which died on the twenty-fourth day, probably from extensive hemorrhage, were opened within a minute after death occurred. None of the fetuses *in utero* was alive. When they were examined in order to determine whether their hearts were beating, they were found to be badly macerated. This is still another indication that the anterior lobe of the pituitary body may be involved. Teel (1926) has found that pregnant rats injected with a sodium-hydroxide extract of the anterior lobe of the pituitary respond in a similar manner, the fetuses dying *in utero*.

When we compare the data secured from the two groups of pregnant rats, we find that the larger dose increases the severity of the reaction. Whether the failure to deliver is caused by the inability of the uterus to contract remains to be determined. It seems possible, however, that in the experimental hyperthyroid rat the failure to parturate may be due to the inability of the oestrus-producing hormone to act on the uterine muscles to produce the normal contractions associated with parturition. The increased rate of metabolism associated with a hyperthyroid condition may cause the oestrus-producing hormone to be so rapidly utilized or removed from the body that not enough is present to cause normal uterine contractions. That this may be true is indicated in the work of Reiss and Perény (1928), who injected oestrus-producing hormone into thyroid-fed female rats and were unable to bring them into heat.

LITERATURE CITED

Chidester, F. E., and W. M. Insko, Jr. 1929. Influence of desiccated thyroid and iodine on growth. II. With a standard acid diet. Amer. Nat., LXIII, 239–47.

Gudernatsch, J. F. 1915*a*. Feeding experiments on rats. Anat. Rec., IX, 78–80.

———. 1915*b*. Feeding experiments on rats. III. A further contribution to the knowledge of organs with an internal secretion. Amer. Jour. Phys., XXXVI, 370–79.

Hoskins, R. G. 1910. Congenital thyroidism: an experimental study of the thyroid in relation to other organs of internal secretion. Amer. Jour. Phys., XXVI, 426–38.

Kunde, M. M.; A. J. Carlson; and T. Proud, 1929. Ovary in experimental hypo- and hyper-thyroidism; influence of experimental hyper-thyroidism on gestation. *Ibid.*, LXXXVIII, 747–53.

Long, J. A., and H. M. Evans, 1922. The oestrous cycle in the rat and its associated phenomena. "Univ. of Calif. Memoirs," VI. 113 pp.

Reiss, M., and S. Perény, 1928. Thyreoideahormon und Brunst. Endokrinologie, II, 181–86.

Teel, H. M., 1926. The effects of injecting anterior hypophysial fluid on the course of gestation in the rat. Amer. Jour. Phys., LXXIX, 170–83.

HONEYDEW REFLEXES

E. A. ANDREWS
Zoölogical Laboratory, Johns Hopkins University

INTRODUCTION

IT WAS Reaumur (1738) who discovered that aphids on trees could be found by the naturalist through following the swarms of ants that visit the aphids; and that the ant does not kill the aphid, as Leeuwenhoek had supposed, but rather seems fond of the aphid, and never kills an aphid as long as it is intact. It has since been demonstrated that ants and aphids form a symbiotic association; and Wheeler (1913), in his classic work on ants, has brought together the reasons for regarding this association as one of mutual advantage.

The action of ants toward many insects is often very complex. For instance, in India, as related by Hinston (1928), certain ants even herd straggling membracids back into the sheds the ants have built about these insects. Or again, some of our native ants have been observed, by an assistant of Forbes (1906), to place aphids upon certain weeds to feed for a few hours and then to take them back to the roots within the ants' nest. Though it seems impossible to reduce such complex actions to simple reflexes, yet there are some interactions of ants and insects producing honeydew that may be regarded as largely of a reflex nature. The evidence for this conclusion is here given as drawn from observations upon certain aphids, membracids, and coccids—chiefly upon membracids.

EVIDENCE FROM APHIDS

The interesting history of honeydew up to the final demonstrations of the origin of much of it from the sucking aphids was related by Buesgen (1891), who also laid the foundation for the understanding of how the insect finds the juice of the plant and draws it out; and this knowledge has been added to by Zweigelt (1915) and by Weber (1928). The way in which the aphids react to the ants and the way the ants play upon the aphids with their antennae was long ago most aptly described by Pierre Huber (1820).

The honeydew from aphids is the end product from the digestive tract; and as it still contains much sugar not utilized by the aphids, it is of great food value to the ant. At the same time its dextrine content adds to its sticky nature and makes it a danger to the aphids themselves if it falls upon them and dries upon them.

The instinct of the aphid to render up to the ant this sweet excretion seemed to Charles Darwin (1884) an apparent difficulty in his effort to derive instincts by natural selections, since there seemed here a case in which the instinct was apparently of no use to the creature that possessed it but only to another species, the ant. However, he finally concluded that this instinct was of some use to the aphid, since it led the ant to remove a material from the aphid.

In Darwin's experiments he removed from a group of aphids all attendant ants; and, after waiting some hours, till the aphids should be ready to give up their secretion, he said: "I tickled them and stroked them with a hair in the same way, as well as I could, as the ants do with their antennae; but not one excreted." Then he placed an ant with the aphids; this played upon the aphids with its antennae, and each aphid at once gave a drop of sweet juice to the ant.

While Darwin did not succeed in mechanically stimulating the aphid to give forth honeydew, and speaks of the act as being voluntary, yet others have succeeded. Thus, Mordwilko (1907) found that aphids touched with a needle gave forth honeydew drops; and Jones (1929) found that gentle touches with hairs, grass, or any other object led to the same results as when the ants "thumb" or "pet" the aphids. The giving-up of honeydew in response to various mechanical stimuli may be interpreted as a reflex act.

Directly comparable to this reflex yield of honeydew are the findings in termites, in which Escherich (1909) states that the experimenter may, with a brush or with light pressure, evoke the same discharge as is normally called forth by the brushing actions of the antennae and palpi of termites about to receive procotodeal food.

I found several sorts of aphids that did not eject honeydew upon stroking with fine hairs, but a small pale aphid on carrot roots in 43 trials on 35 individuals gave 7 positive and 36 negative results.

The positive results were on two separate individuals, one of which had been kept isolated from ants and gave 1 drop when stimulated by a dry leg of an ant held on a needle, while the other gave 6 drops in 1.25 minutes when struck with a single hair of a kitten, at rates varying from 15 to 20 strokes per minute. But the same aphid gave forth no drops through 2 minutes when left without stimulation. Hence, stimulation increased the frequency of drops, confirming Mordwilko (1907), who found that aphids with no ants threw off few drops at long intervals but, when stroked by ants, gave off many drops, each slowly.

In a cluster of these aphids not stimulated artificially or by ants, one or the other from time to time will present a clear drop of honeydew at the anus; and then, if this be not taken off by an ant, this aphid, though but 1 mm. in length, will project it with such force that it travels in a curve as much as 1 cm.; and this drop, after falling 2 cm., if caught on a cover glass, spreads out as a mass $\frac{1}{2}$–1 mm. wide. Thus, in the absence of external stimulation, the aphid does cast off the sticky excreta from its body; but in crowded communities drops are found to have fallen on the backs of other individuals, so that the contention of Darwin, that the aphid, in yielding honeydew to the ant, instinctively betters the conditions for its own species, may still be maintained.

EVIDENCE FROM MEMBRACIDS

As elsewhere related (Andrews, 1929), I found that a certain ant, *Formica exsectoides* F., obtains much energy-yielding food from honeydew products of the young stages of two tree-hoppers, *Thelia bimaculata* Fab. and *Vanduzea aquata* Say. This association of these two membracids and this ant was studied not only in the field but near an ant mound transplanted and maintained for some years in the university [Johns Hopkins] grounds, as well as upon small trees transplanted into the laboratory, inoculated with the membracids and attended by ants kept in various large and small termitaria.

These two membracids, like their tropical relatives, have the following complex habit in discharging honeydew without external stimulus. By action of leg and body muscles the rear body is raised up from the tree bark, then turned to the right or to the left and elongated. Then from the blunt end there emerges more and more a long tubular continuation strikingly unlike the rest of the body in color, the tip being black and final base, where joining the usual body, clear white and translucent, so that the microscope reveals the digestive tube with drops passing along peristaltically toward the anus, which is at the extreme end of the elongated tubular body ending. A drop issuing from the anus is held in a basket of setae but immediately projected off into space, to be quickly followed by few or many drops in a fountain-like outburst. Thereupon the anal tube is withdrawn into the usual body ending and the normal posture of the body resumed. The force of projection is such that from a *Thelia* larva drops travel 3.5 cm. horizontally and 1.5 cm. vertically, and the amount cast off in one fountain discharge, accumulates on a cover glass as a pool 5×3 mm. and $\frac{1}{2}$ mm. deep. In the smaller *Vanduzea* the drops are much smaller. We will speak of the appearance of a drop as "presentation" and its throwing-off as "projection."

This complete chain of actions is often started and then checked, not carried through to actual presentation of drop, much less projection. On the other hand, a larva, while free to get up and walk about and settle down in some other place, often remains hours or days in one spot. One exhibited remarkable accuracy in the repetition of its acts, turning the anal tube and body to one particular side, time after time, and then projecting the honeydew with mechanical precision so that each fountain of droplets falling upon a glass that was slowly drawn along (1 hour and 40 minutes) was exactly imitated in size and distribution of component drops over and over again. Both the aim of body and legs and the force of contraction of the rectum were thus exactly repeated. However, the intervals between projections are very diverse in one individual and in the various members of a group, so that one cannot predict when any one insect will emit drops.

While the fountain of drops normally arises from unknown internal conditions that start contractions of both body and rectum muscles, all these actions may be called into play from the external stimulus given by a slight blow upon the twig holding groups of *Vanduzea*. Then one or the other of several larvae in the different groups will project drops into space. Three of four in a group of twenty may project. However, some larvae do not react, and some merely *present* drops.

Contrasted with these complete actions that project the honeydew violently are the modified reflexes when ants or artificially applied mechanical stimulations lead to presentation of drops that are held till removed, or else finally drawn back into the anal tube.

When a *Vanduzea* larva, 1½–3 mm. long, or a larger larva of *Thelia*, is picked up with fine forceps, it generally rears up the abdomen, points it to one side, evaginates the anal tube, and presents a drop of honeydew which may come off on the forceps or be held from many seconds up to 1.4 minutes. Similar reflex presentation follows the piercing of the body of *Vanduzea* larva with a fine needle. Moreover, these larvae may be played upon by seizing, with fine forceps, the tip of each leg in any sequence, whereupon the body is aimed toward the side of stimulation and the presented drop often adheres to the forceps. Thus, a tweak to a right or left leg (first, second, or third) was followed by an aimed reflex presentation of a drop to the right or to the left, respectively. Pressure of a blunt needle on a foot led to the discharge of a drop toward the side stimulated. Even on a table, removed from food supply, *Vanduzea* larva gave 3 drops alternately as left or right leg was taken hold of.

These modified reflex presentations of honeydew recall responses of a reflex frog and also raise the question whether they are not in the nature of useful defense reactions. It is such modified reflex presentations that enable the ants to drink honeydew direct from the anal tube, in addition to licking up the drops discharged in fountains.

But that the ants, when attending these membracids, get honeydew with the aid of these reflexes is not entirely self-evident. This kind of ant, running along the branches inhabited by the sucking insects, may pass rapidly from group to group or may, in other conditions, remain long, even all night, with one group. In either case the ant goes from insect to insect, playing upon them with its antennae and palps. But the presentation of honeydew does not follow every time the ant plays upon an insect; and, on the other hand, the insect may present a drop when not recently approached by an ant, hold it, and, if not taken, draw it back into the tube. Moreover, some few of the insects that are attended by ants may cast off drops as if not externally stimulated. It might be that the ants, examining many insects, chanced to find one that presented a drop here and there and was about to cast it forth, had not the ant drunk it up.

It can, however, be shown that the attendance of ants leads to presentation at frequent intervals, as compared with the fountain projection at long intervals. Thus, when a large *Thelia* larva was isolated from ants and closely observed for 1 hour and 40 minutes, it gave forth projections of from 2 to 6 drops each, at very irregular intervals, 6 projections in the first hour and 4 in the next 40 minutes, or 10 projections in the $1\frac{2}{3}$ hours, from 3:00 to 3:40 P.M. That same *Thelia*, when attended by ants from 11:30 A.M. to 12:30 P.M., made at very irregular intervals 17 presentations of drops that were taken by ants, in one case 6 drops, in another 5 (but the other 15 of unknown number). For this larva, then, there was no evidence that the amount of honeydew was different; but the frequency of discharge was much greater when ants attended, there being only 10 projections in 1 hour and 40 minutes as against 17 presentations within 1 hour.

When these ants visit groups of *Vanduzea* larvae and run away, there seems still some indication that drops are hastened by the interrupted presence of the ants. In one case six larvae gave 26 drops in 2 hours, of which 16 were to ants that came 25 times at the following intervals, in minutes: 1, 1, 1, 2, 2, 1, 6, 3, 1, 9, 2, 2, 2, 1, 1, 1, 17, 1, 1, 1, 10, 1, 2, 2, and remained only a minute or two. Here the drops to ants emerged at intervals of 12, 13, 19, 2, 12, minutes, and the spontaneous discharges at intervals of 14, 1, 32, 2, 36, 10, minutes—the average of the former being 12 and of the latter 16 minutes.

The black ant, *Formica fusca* var. *subaenescens* Emory, invaded experimental trees and obtained honeydew from *Vanduzea* larvae in the same way as *Formica exsectoides*. With the ants going and coming rapidly, a group of three larvae watched for 1 hour and 50 minutes gave 30 drops, that were all taken off by ants; also the intervals between visits were apparently too short for any spontaneous projection; they were, in 57 visits, 7 of ½ minute, 28 of 1 minute, 13 of 2 minutes, 5 of 3 minutes, 2 of 4 minutes, and one each of 5 and 6 minutes. This kind of ant also may examine all the larvae and get nothing, or one or more drops before leaving. Also, the larvae presented drops that were withdrawn again when not removed by visiting ants. With this interrupted presence of ants apparently stimulating the larvae with their antennae, the three larvae presented drops at diverse intervals. One gave 6 drops at intervals of 21½, 3½, 33, 6, 2, minutes; one gave no drops at all; and the third gave drops at intervals of 1½, 4½, 2½. ½, 3½, 8½, 4, 1, 5, 6, 1, 1, 4, 2, 2, 1, 12, 5, minutes.

While the larvae give most of the drops, the adults of both *Thelia* and *Vanduzea* give off drops that often may be caught under the projecting wings till taken by an ant of either species or discharged onto bark. Also, the ants play over the adults with their antennae; but it is not known if the adults are thus stimulated, though their frequent presentations when attended by ants suggests that such may be the case. Thus a group of ten *Vanduzea*, one male, one female, and eight larvae of different instars, attended by the foregoing black ants, gave 31 drops to ants in 57 minutes. The female gave to ants 2 drops, then after 3 minutes 1 drop, after 2 minutes 1 drop, after 20 minutes 1 drop, after 8 minutes 1 drop, after 4 minutes 1 drop, after 5 minutes 3 drops—10 drops in all. One larva gave single drops at intervals of 36, 4, 3, 3, minutes. Another at 5, 40, and 5 minutes; but during the long interval of 40 minutes when no ants were present, it spontaneously projected 4 drops, the first 10 minutes after supplying an ant, the others in 5, then 4, then 10 minutes. A third larva gave drops to the ants at intervals of 8, 11, 11, 5, minutes. The next larva, at intervals of 41, 4, 3, 4, minutes; but in the interval of 41 minutes this larva spontaneously cast off a drop 27 minutes after giving a drop to an ant and 11 minutes before giving the next drop to an ant. The largest larva gave only 1 drop during all the 57 minutes, and that was 46 minutes from the beginning and 11 minutes before the end of that period. One larva gave 1 drop in 2 minutes and then no more. The male adult was not recorded. Two larvae gave nothing to ants; but each produced 1 drop spontaneously, the one in 23 minutes, the other in 15 minutes. Although we have here the greatest diversity in intervals, on the average the intervals between spontaneous projections was 13 minutes and between presentations after stimulations 8 minutes. There were but 7 spontaneous projections and 31 presentations after stimulations.

In such observations on the black ant and *Vanduzea* there is no indication that the ants stimulate the *Vanduzea* in any precise way for each drop given. However, since 31 drops were offered and taken, as opposed to only 7 drops (or fountains) not got by ants, it is evident that the ants were almost always there when the drops were offered and that, furthermore, most of the larvae held their drops and did not cast them forth, so that it is probable that the condition of the *Vanduzea* was modified by the coming and going of the ants, their running among and over the *Vanduzea*, and their playing upon them with antennae and palps.

The playing of the antennae of the ant is a rapid brushing (judged to be 120–400 strokes per minute) from outward, backward, and toward the middle of the ant with the tips of the antennae converging so that both may touch a small object. The antennae tips may be 4 mm. apart naturally. These motions may be made upon the *Vanduzea* or the *Thelia* not only at the anal end but over all parts. That the antennae brush the surface is indicated by the removal of starch and of soda placed on the larvae and brushed off on antennae and also removed by the palps and taken into the mouth when the ant attacked the powder. Biting on the powder led to the presentation of honeydew, which the ants took even through the concealing powder. However, prolonged attacks by an ant led the larvae to move away.

A young larva, as soon as touched by the antennae of an ant, may rear up and present a drop; but, as stated above, this often is not the case. Assuming that both these two kinds of ants may excite the *Vanduzeas* and *Thelias* to present honeydew by stroking with their antennae rather than by foot movements or palp movements, we endeavored to stimulate these larvae with fine hairs, singly or in small brushes, also with the antennae and the feet of dead ants.

Fifteen minutes after removal of black ants from *Vanduzea* a large larva was struck with a small brush of hair of a kitten. The larva responded for each stroke by throwing out the anal tube part way; but with rough brushing, it protruded the tube full length and presented a drop, and again in less than a minute the brushing led to a second drop.

Seven larvae, struck at the rate of 40–120 times per minute with the antennae of a drowned *Formica exsectoides*, projected the anal tube 20 times out of 25 trials. Two larvae stimulated by a single hair threw the body violently from side to side and then walked away. A brush of fine bat hair loaded with starch, with 50 to 100 strokes per minute, was followed by 5 protrusions out of 7 trials. A brush of kitten hairs led to such protrusions and also to 1 display of honeydew.

In the larger larvae of *Thelia*, however, responses to mechanical contacts were much more pronounced.

Thelia larvae placed upon small locust trees in the laboratory were struck by hairs of a kitten and of a bat, one or a few in a brush, and by antennae and legs of dead ants, *Formica exsectoides*. While most strokes were on the back and the posterior end, some were on the head. In all cases there was protrusion of the anal tube; and in a few, drops were presented. Presented drops were taken back into tube when not removed by needle. The larvae took up isolated positions on a vertical stem, and many remained stationary—one 13 days. During this period it was stimulated 56 times, with 9 failures and 47 positive results (and then walked away, after stimulation). In all, twelve larvae were stimulated 199 times. In 146 of the times there was protrusion of the anal tube; in 53, there was no response.

The individual diversities of these twelve larvae are reported as follows, the number of successes being followed by the failures in each of the twelve larvae: 8, 1; 3, 5; 2, 2; 11, 1; 11, 1; 25, 12; 2, 2; 2, 2; 14, 13; 47, 9; 9, 3; 10, 2.

Ten *Thelia* larvae were left alone 14 minutes, during which three of them cast off drops; and then in a second period of 9 minutes, one of the two cast off and a fourth likewise. Then for 12 minutes these same larvae were each in succession given rapid strokes by a brush, of probably twenty kitten hairs, during which treatment all but one of the ten responded with projection of anal tube; while in continuing the treatment another 16 minutes, all but one (a different one) responded, and that one was uncertain, being partly concealed from view. Thus there were 90 per cent of responses to these long-continued but interrupted successive stimulations.

In the following 8 minutes but one hair was used and only five larvae responded, and in the next 10 minutes only two responded, one of these being the one that responded in the 8-minute period. Apparently the brush with many fine contacts long-repeated was more efficient as stimulus than the single hair, but this one hair led to more partial acts than there were complete discharges when there was no external stimulus.

Yet a single hair struck upon a 6-mm. larva at the rate of about 200 strokes per minute for less than ½ minute was followed by a protruded drop that was licked off by a passing ant. When the treatment was continued, a second drop came off onto the needle holding the hair, and then a third drop onto the needle holding a brush of many hairs. Several older larvae near by failed to reply at all to the like stimulus.

As bearing upon a probable advantage of numerous contacts obtained by using a brush may be cited the following further experiment: Twenty-seven larvae with a brush gave 81 per cent protrusions; with only 2–8 hairs, twenty-one gave 52 per cent protrusions; yet with a single hair, forty-one gave 86 per cent protrusions. When the much finer hairs of a bat were used, one hair 5 mm. long induced 40 per cent responses from nineteen larvae; while a brush of these hairs induced 41 per cent responses in twelve larvae. These hairs are so fine that one was but slightly felt touching the forehead between the eyes of the observer.

The duration of contacts before response was most varied. When a *Thelia* larva was beat upon with the antennae of a dead *F. exsectiodes*, there was protrusion of the anal tube in from 0.1 to 1.2 minutes in 10 trials that varied in rate of rapidity of stroke from 80 to 200 per minute, but there seemed no correlation between the speed of the stroke and the length of time before response. Another larva struck in 5 trials responded in from 1.5 to 0.8 of a minute when the rate varied from 116 to 162 strokes per minute. Failure to protrude followed 3 trials of from 0.6 to 1.5 of a minute with rates from 68 to 165 strokes per minute.

In general, while the stimulus seemed to be the summation of successive contacts, it was not necessary to continue the brushing more than 1 minute before protrusions followed; while without any attempted stimulation, the larvae remained many minutes without any visible changes.

The increased frequency of activity of the larvae when brushed was shown when ten *Thelia* were observed during 34 minutes of no external stimulation to make 4 discharges of 44 drops; while, when brushed during the next 34 minutes, they exhibited 15 protrusions though but 1 drop.

That the quickness of response of a particular larva may be increased is indicated by the observation that a *Thelia* larva struck with antennae of a dead ant protruded the anal tube after 1.3 minutes, during which it was struck 150 times. But the next day, after having its right front foot bitten off by a raging ant, this larva, when struck with the same antennae, protruded the anal tube in 0.3 minute when struck but 19 times, in 0.1 minute when struck but 2 times, in 0.05 when struck but 2 times, in 0.1 when struck 8 times, and then in 0.2 when struck 17 times. When, then, passing ants went near it, it also everted its anal tube, though not played upon by their antennae. A day later, however, it was less irritable, not responding when struck 100 times during a minute with the leg of an ant. But 40 minutes later it was able to cast off spontaneous drops, and held out a drop for several seconds after the passing of an ant. With other larvae the irritability induced by brushing seemed to lead them to offer drops readily to passing ants even when the ants did not stop to play upon them with their antennae.

EVIDENCE FROM THE ACTIONS OF CERTAIN FLIES

It is not only the ant that makes use of the modified reflex of these insects. The reflex responses of *Vanduzea* larvae are utilized by certain sugar flies,[1] several species of which search for and lick up the fallen honeydew while at least two species also drink from the font directly. One of these flies, *Revellia quadrifuscata* Macquart, when not driven off by *Formica exsectoides*, rapidly runs up behind the larva and actually drums 6 or more times per second with its two front feet, alternately, upon the end of the larva; and very generally a drop is protruded quickly and sucked up by the proboscis of the fly. Here again, we seem to be dealing with a state of widespread irritability among the larvae, for a fly may drum upon one larva and drink 4 drops in quick succession, then turn aside and drink 2 drops from another larva, come back to the first larva, play upon it with its front legs, get 2 more small drops, all in a short moment. The mechanical stimulation applied by this fly seems most efficient.

EVIDENCE FROM CERTAIN COCCIDS

Some observations upon coccids, or scale insects, also indicate that ants here take advantage of a modified reflex. While in the foregoing membracids, as in tropical members of this group, the reactions to ants are more complex and involve special anatomical formations of the abdomen, in the coccids the honeydew is given to ants in a more simple way. The ant *Formica exsectoides* obtains very large supplies of honeydew from the common scale insects upon the tulip-poplar trees, and apparently by stimulating reflex discharges, made as from aphids. The ants find these scale insects dispersed along under the branches, where each scale insect sits separately in one spot, and permanently so during its life.

In general, there is thus a procession of ants going and coming past the scales, stopping at frequent intervals to receive honeydew, and passing on. Each scale is an inert, irregular conical mass, showing no sign of life except the occasional opening of a minute dorsal lengthwise slit between two elevations. From this we see a minute drop of honeydew slowly emerge and grow larger for about 1 second, then rest as in a shallow cup, but suddenly it will be cast far from the animal without any other probable cause than the contraction of the rectum. Such drops are sweet and soon form a sticky varnish upon objects beneath the limb, from which a slow shower of drops may be seen against the sun, about 1 per second from a length of 1 foot of a small branch.

Ants intercept many of these drops before they are cast off, so that the ant soon becomes replete and leaves the tree, weighing

13 mg. as compared with its weight of 9–10 mg. when coming to the tree.

Having cut off ant-access by walls of vaseline, three scales were watched for 30 minutes. One cast no drops; one cast 1 drop after 17 minutes and no more, and the other cast 6 drops at the following intervals: 10.5, 2.5, 0.5, 2.90, 1.85, 3.75, minutes.

Contrasted with the preceding rate of discharge when no ants were present, we observed a scale in the normal state, with ants coming and going. During 20 minutes this scale gave 20 drops to ants, and each ant that came got a drop, except in 4 cases. The time consumed in taking a drop varied from 0.05 to 0.60 minutes. The ants arrived at the following irregular intervals and got drops, that is, the scale presented drops at the following irregular intervals: 0.5, 1.10, 2.10, 2.50, 2.60, 0.01, 0.01, 0.09, 0.09, 0.12, 0.05, 0.15, 0.10, 0.10, 0.02, 0.08, 0.10, 0.15, 0.30 minutes. There were, however, 13 intervals of from 0.01 to 2.60 minutes when this scale was left without any ant being present, and in one of these the scale presented a drop for 0.65 minutes before it let it fall, and later it presented a drop for 0.20 minutes before an ant came and took it. The interval between these two spontaneous discharges was 1.35 minutes. And this interval was long in comparison with most of the intervals between drops given to ants—in fact, longer than the average, though the intervals between drops given to ants were at first longer. The fact that the scale speeded up the presentation of drops as the ants came more frequently shows the correlation of presence of ants and discharge of honeydew.

Another scale observed through 2 hours also showed a difference between occasional spontaneous discharge and frequent supplies to ants. For, when the times that ants were present and the times when ants were not present were plotted, it was seen at once that, while there were many long intervals with no ants, there were few long intervals with ants present; and, also, that the drops given off did not correlate with the lengths of time but with the presence of ants, 1 or more drops being put out by the scale insect almost every time when ants were present and very few given off in the 19 long intervals without ants. The ants came to the scale 50 times in the 2 hours and got from 1 to 9 drops each time, an average of

2 drops for each time present, though they went away without any drops in 9 cases.

The length of time the ants remained varied from 0.05 to 2.30 minutes. Twelve ants obtained each a drop in the brief visit of about 3 seconds. There were 33 intervals when no ants were present; and, though these intervals ran from 0.1 to 3.60 minutes, drops were not given off except in 2 cases—1 drop at the end of the extreme period of 3.60 minutes and 4 drops at the end of a few minutes. Thus, with no ants present for a total of 18 minutes, only 5 drops were produced by this scale, while 103 drops were produced in the total of 10 minutes when ants were present, although no drops were produced during 4 minutes of this time.

On the 9 occasions when ants came and went away empty, it was observed that on 1 occasion the ant played upon the head end of the larva, on 3 occasions the ant ran over the larva or upon its margin, and only on 5 occasions did the ant play upon the dome of the larva with its antennae, as was done in all the occasions when the ants actually obtained drops. The actual visits that were successful in obtaining drops graded from 0.05 to 1.40 minutes. Visits that lasted up to 2.30 minutes without drops were spent by the ants walking upon the scale but not playing upon it with their antennae.

Observations suggested that the ants generally did not know when or where the drops were to emerge till feeling over the dome of the scale, and that apparently the scale was thus stimulated to put out the honeydew soon after the arrival of the ant.

Assuming that the ants stimulate the scale to emit honeydew, attempts to produce this response by mechanical stimulation with hairs were made, as in the case of the foregoing membracids. Striking during 12 minutes led to no response. Many strokes with the antennae of a freshly decapitated ant produced no visible result, though a live ant came up and, after playing upon the scale, received 3 or 4 drops in quick succession. Yet, with two hairs of kitten a few drops were got from two other scales, but these drops may have been spontaneous. While in this scale there is evidence that the ants modify the rate and the character of the honeydew discharge, taking presented drops after antennal play, which drops would usually have been projected but for the ant, the evidence that this is a reflex due to stimulation by the ants' antennae is less satisfactory than in the case of the membracids, since mechanical stimulation by hairs did not generally induce drops.

GENERAL CONCLUSIONS

Aphids, membracids, and some coccids spontaneously cast off honeydew with great muscular effort; and as they are sedentary or of sluggish habit when larvae, these muscular efforts are relatively important.

By symbiosis with ants they obtain a more complete removal of adhesive material injurious to the colony than could be got even by their greatest efforts, and by modifying their reflexes they yield the honeydew direct to the ants with minimum of effort. Moreover, the presence of ants that are on the defensive probably affords some protection to the insects thus guarded. These modified reflexes are thus of threefold advantage: better disposal, less effort, and some protection.

Of these apparent advantages, the conservation of muscular work when the insect responds to ants seems not to have attracted the attention of previous observers.

Apparently it is a summation of slight stimuli that puts the insect into an irritable state. It is not that the ant, by touching the honeydew insect, stimulates it at once to give out honeydew, but that, by frequent stimuli, it sets up a state of tension leading to frequent modified discharges. Jones (1929) states that aphids constantly in care of ants continue to emit the honeydew very slowly at all times, even when the ants have been kept from them long enough to allow the excrement to accumulate in their bodies in large quantities.

Possibly these modified reflexes may have arisen from an association of discharge with defense against enemies, severe attack stimulating the nervous system to carry out one of its most fundamental complex actions by a widespread distribution of innervation. By modification of such complete response, eventually very slight stimulation might have led to the initial stages of a complete discharge; and by an inhibition of the final effort, an end result was got, in which the ant substitutes for the severe work of the rectum in projecting the honeydew.

Having observed the actions of membracids attended by certain ants, Wheeler (1913) stated: "The Homoptera responded to the antennal caresses of the ants in precisely the same manner as plant-lice and scale-insects." It is this manner which we have ventured to speak of as reflex, since it may be called into action by various mechanical stimuli.

The manner of action of the ant in getting the honeydew may also, possibly, be looked at from the point of view of reflexes.

Wheeler (1913), speaking of ants, says: "Many species have learned to stroke the aphids and induce them to void the liquid gradually so that it can be imbibed directly." What induces them to stroke the aphids has not been found out. It is well known that the ants may attend aphids and stand on guard as if dealing with their own young, and Funkhouser (1915) was struck by the habit of some ants to stand at the head end of the *Thelia* as if drinking escaped honeydew. I also observed that *Formica exsectoides* went carefully over the bark that had been under the body of a larva that moved away, and also that, when the membracid *Vanduzea* shed its skin, these ants greedily investigated the inside of the cast skin. In fact, so great is the interest of some ants in the membracids when shedding, that one observer published the statement that the *Vanduzea* could not shed without the aid of the ants. May it not be that all this attraction exerted by these membracids and their skins and the spots where they have long stood may be due to some secretion from the body that stimulates the ants which are thus drawn to the membracids by stimulation of their topo-chemical sense organs? Wheeler (1913) has said that "it is probable that the aphids and coccids make the same appeal to the ants' sense of ownership as their own larvae and pupae." Now it is generally understood that much of the interplay of ants among ants is based upon topo-chemical responses; and if this be also true for their actions toward the givers of honey dew, it may be here also that reflexes called out in the ant by odors and forms of these insects are the main basis for their attendance, and their ultimate acquisition of honeydew a secondary incident, arising when the honeydew is actually presented, or on the way to the vent, and then first sensed as honeydew.

This point of view is in harmony with the statement of Jones (1929), who, after having observed a great many kinds of ants attending very many sorts of aphids, came to the conclusion that the ants' antennae move the same when the ant is feeling a stone in its path as when stroking an aphid; and he concludes that the so-called "thumbing" or "petting" movements of the antennae of an ant upon the aphid are not for the purpose of causing the aphid to emit the sweetened excrement.

SUMMARY AND ABSTRACT

1. Without any external stimulus, certain aphids, the membracids *Thelia bimaculata* Fab. and *Vanduzea arquata* Say, as well as the tulip-tree scale, will, from time to time, project honeydew from their bodies by a violent muscular effort.

2. Certain external mechanical stimuli may lead to the like result. However, gentle, reiterated stimuli lead to a modified result: the drop of honeydew is presented and held in a sort of basket till removed by outside forces, or else withdrawn back into the body again.

3. The commonly accepted conclusion that ants, by movement of their antennae upon the honeydew producer, elicit the modified flow of honeydew becomes demonstrated by the facts that, first, when ants attend these insects, the intervals between presented drops are shorter than the intervals between spontaneous projections, and second, that various artificially applied mechanical stimulations induce the same modified presentations of honeydew.

4. The symbiotic adjustment of these sucking insects to the ants is due to their reflexes having become adjusted to slight external mechanical stimuli applied by the ants.

5. The symbiotic adaptations of these ants to the producers of honeydew may also be based upon reflexes—topo-chemical stimulations arising from assumed secretions of the skins of these insects. The drinking of honeydew is not necessarily foreseen by the ant.

6. Certain sugar flies actually apply mechanical stimuli to some membracids through violent movements of their forelegs, and thus liberate and profit by the modified reflex.

LITERATURE CITED

Andrews, E. A. 1929. The mound-building ant, *Formica exsectoides* F. associated with tree-hoppers. Amer. Ent. Soc. Ann., XXII, 369–91.

Buesgen, M. 1891. Der Honigtau. Biologischen Studien an Pflanzen und Pflanzenlausen. Jenaische Ztschr., XXV, 339–426.

Darwin, Charles. 1884. The origin of species by means of natural selection (6th ed.), p. 207. London.

Escherich, K. 1909. Die Termiten. Leipzig.

Forbes, S. A. 1906. The corn root aphis and its attendant ant. U.S. Dept. Agri. Bur. of Ent., LX, 29–41.

Funkhauser, W. D. 1915. Life history of *Thelia bimaculata* Fal. Amer. Ent. Soc. Ann., VIII, 140–52.

Hinston, R. W. G. 1928. Problems of instinct and intelligence. Macmillan.

Huber, Pierre. 1820. Natural history of ants. Transl. London.

Jones, Charles H. 1929. Ants and their relation to aphids. Exp. Station, Col. Agri. College, Bull. 341.

Mordwilko, A. 1907. Die Ameisen und Blattläuse in ihren gegenseitigen Beziehungen und das Zusammenleben von Lebewesen überhaupt. Biol. Centralbl., XXVII, 212–24, 233–52.

Reaumur, R.-A. 1738. Mémoires pour servir à l'histoire des insectes, III, 312.

Weber, Hermann. 1928. Skelett, Muskulatur, und Darm der schwarzen Blattlaus, *Aphis fabae* Scop. Zoologica, XXVIII, 1–120.

Wheeler, W. M. 1913. Ants. New York: Columbia University Press.

Zweigelt, Fritz. 1915. Beiträge zur Kenntnis des Saugphänomens der Blattläuse und der Reaktionen der Pflanzenzellen. Cbl. für Bakteriologie, Parasitenkunde und Infectionskrankheiten, XLII, 265–335.

A STUDY OF CHITIN SECRETION IN DAPHNIA MAGNA

(Five figures)

B. G. ANDERSON AND L. A. BROWN
Zoölogical Laboratory, State University of Iowa

IT IS well established for certain arthropods that chitin for the new exoskeleton is secreted before the old one is cast off. Jackson (1890) in his studies on *Vanessa* noted two layers of chitin immediately prior to molting, but considered the inner one a fluid which had coagulated on preservation. Hesse and Doflein (1910) in a brief description of ecdysis in the crayfish (*Potamobius asticus* L.) show that in its growth the organism arrives at a certain stage in which the old chitinous coat loosens from the hypodermis and a new coat is immediately secreted. After secretion has continued for a time the old coat is cast off.

Two distinct layers of chitin can be observed microscopically in stained and teased preparations of *Daphnia* killed late in the instar. Early in the instar but one layer is found. By killing *Daphnia* in hot corrosive-sublimate solution the writers were able to determine the degree of adherence of the chitin to the underlying tissue. In an animal which has recently molted (within 24 hours at room temperature) the chitin remains attached to the underlying layers when submitted to this treatment, while in later stages of the instar (within 24 hours of the end of the instar at room temperature) the chitin separates completely from the underlying tissue.

When the carapace of *Daphnia magna* is damaged, a substance forms around the injured portion and later turns brown. A similar condition is described by Andrews (1906) for the crayfish (*Cambarus*), in which he removed the sperm receptacle and found there was formed a membrane which turned brown. At molting this membrane was cast off with the old shell, and it was noted that it was in intimate contact with the chitin at all its edges.

In *Daphnia* the brown material is sometimes shed at the first ecdysis following the injury and at other times only at the second. The loss or retention of this brown substance at the first ecdysis is related to the time within the instar that the injury was inflicted. Brown material from injuries produced early in the instar is shed at the next molt, but that from injuries produced late is retained until the end of the following instar.

These facts seem to indicate that the retention of the brown material is directly related to the presence of a second layer of chitin at the time of injury. Therefore, by injuring the animal at definite intervals within the instar and observing the character of the wound area during the postoperative instar, the time at which two layers of chitin are present might be determined.

MATERIALS AND METHODS

The material used, *Daphnia magna* Straus. var. schaefferi, may be traced to Banta's and on to MacArthur's stocks (MacArthur and Baillie, 1929). Females from one clone, started in January, 1929, were used exclusively, thus insuring animals genotypical throughout. Individuals were isolated within 24 hours after their release from the mother and placed in vials containing 30–35 cc. of manure-soil medium (Banta, 1921) and kept at room temperature until sexual maturity, when they were placed in a Frigidaire fitted with a mercury thermostat and an electric fan (air temperature ±0.5° C.). No food material or water was added to the vials during the course of the experiments. The broods released, however, were large enough (five to fifteen young to a brood) to insure that the medium was satisfactory. In order to allow for acclimatization, 2 days were permitted to elapse after placing the animals in the Frigidaire before observations were begun.

In the operative procedure the animals were placed in watch glasses and by removing the excess liquid were immobilized. A small and definite area of the carapace in contact with the watch glass was crushed by applying a ball-pointed steel needle to its inner surface. Practically no mortality resulted. The brown material forms around the region of injury in a few hours. Microscopic examinations of the animals and their cast carapaces were made and recorded within 24 hours after the end of the instar.

EXPERIMENTAL

Microscopic examination of the carapace of every individual during the instar following the operations showed three classes of wounds represented by Figure 1, *A*, *B*, *C*. These differ from each other in the markings of the chitin, in the character of the inner edge of the wound area, and in the presence or absence of the brown material. The more or less regular rectangular spaces surrounding the wound area are the typical surface markings of the entire carapace of this species of cladoceran.

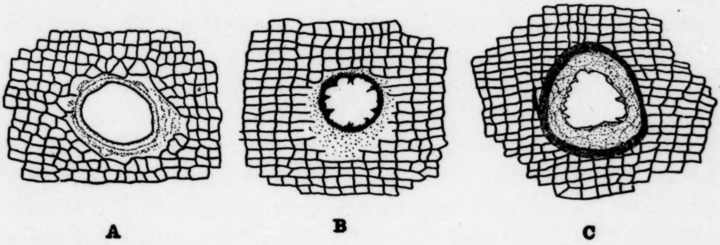


FIG. 1.—Camera-lucida drawings of wounds during the first postoperative instars.

Figure 1, *A*, shows a wound made when 40 per cent of the instar had passed. No chitin had been secreted before the time of injury. The chitin on the inner edge appears clear and smooth (stippled area). The irregular markings (just outside of the stippled portion) are typical after regeneration of the hypodermis has occurred. These are different from the characteristic rectangular markings of the uninjured carapace (outermost markings).

Figure 1, *B*, shows a wound inflicted after 62 per cent of the instar was over. The inner edge of the wound area is rough and frayed. The markings of the carapace are typical throughout. Chitin secretion had just begun at the time of injury. Enough had not been secreted to retain the brown material during the instar after injury, as is the case in the following figure.

Figure 1, *C*, shows a wound resulting from injury made when 75 per cent of the instar was completed. The brown material (closely stippled portion) was carried over from the previous instar. The markings are regular throughout.

In Figure 1, *A*, from a wound produced when 40 per cent of the instar had passed, the inner edge of the wound is smooth and clear. The markings of the chitin bordering this area are irregular and are not typical of an uninjured area, as may be seen on comparison with the outermost markings. This is a type of modification which is found after wound closure has occurred. A similar condition is found in the second instar after operation except that in such cases the odd markings cover a greater area. No chitin could have been secreted before the time of injury as this corresponds to the time within the instar when the chitin coat was not loosened from the underlying tissue by submersion in boiling corrosive-sublimate solution.

In Figure 1, *C*, from a wound produced when 75 per cent of the instar was over, the brown material was carried over from the previous instar. The time of operation in this instance corresponds to the cases in which two chitin layers were found upon microscopic examination and when submersion in hot corrosive-sublimate solution caused a separation of the chitin from the tissue. Evidently the brown material on forming must have become attached to the inner edge of the chitin. The brown material has never been observed to remain with the animal for more than one instar after injury. In another series of experiments concerned with regeneration carried on by one of the writers (B. G. A.), in which the same operative technique was used in over two thousand cases, no such phenomenon occurred. Andrews (1906) noted that in the crayfish the brown material was shed with the cast shells on the first molt after operation, but he gives no data pertaining to the relation of the time of operation and molting.

Figure 1, *B*, a wound made when 62 per cent of the instar had passed, shows no changed configuration of the chitin, indicating that no closure of the wound took place between the time of operation and the beginning of chitin secretion. The ring of brown material (as found in Fig. 1, *C*) is not present. The second layer of chitin, if secreted, was not sufficient to carry over the brown material into the postoperative instar. The edge of the wound is rough, and hairlike processes are present which may be of chitin and may have been secreted previous to the time of operation. If so, they must be of the first chitin secreted, and such secretion must have begun but a short time before the time of operation. This figure represents an intermediate stage since it has neither the smooth clear character and the changed configuration of the chitin as shown in Figure 1, *A*, nor the brown ring shown in Figure 1, *C*. Operative times which produce wounds of this type are taken as the time of the beginning of chitin secretion.

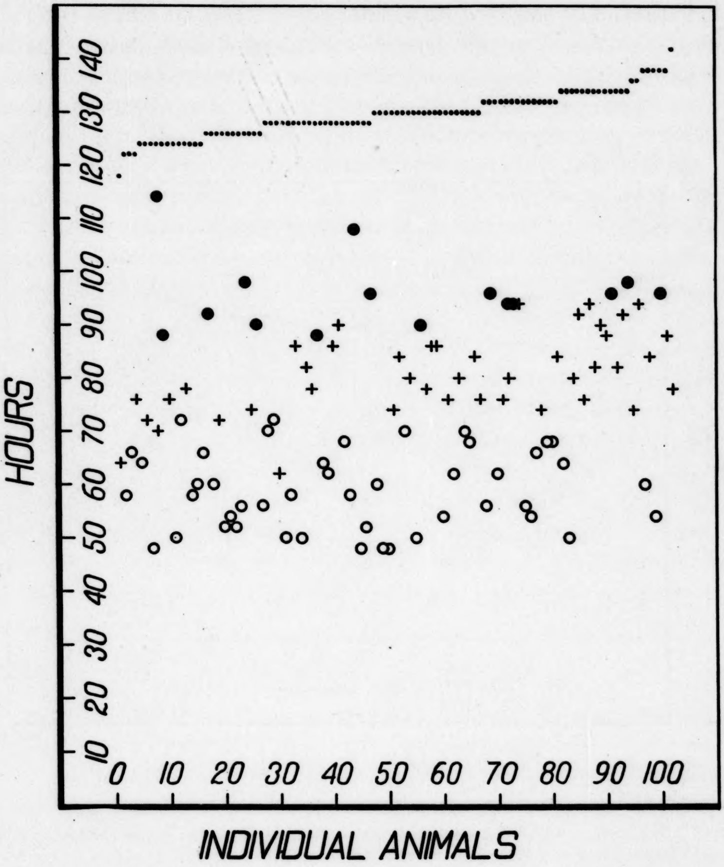


FIG. 2.—A graph showing instar lengths and operative hours for the experiment at 15° C. The base line corresponds to the beginning of the instar for each individual animal. The small solid circles represent instar lengths. The large solid circles represent times of operation which produced wounds resembling Figure 1, *C;* the crosses, Figure 1, *B;* and the open circles, Figure 1, *A*. The mean instar length was 129.53 ± 0.28 hours for the 102 operated animals and 130.97 ± 0.34 hours for the 41 unoperated controls. For animals displaying Class B wounds the instar length was 130.54 ± 0.48 for 43 cases. The mean operative time in per cent of individual instar lengths of the latter animals was 61.68 ± 0.57.

Figures 2, 3, and 4 show graphically the relation of the type of wound produced to the operative hour and also the instar length of each animal in experiments carried on at three different temperatures. The operations giving Class A wounds come definitely at an earlier hour than those of Class B, while those of Class C come clearly at a later time than those of Class B. From a study of these graphs it is readily seen that chitin secretion begins at a fairly uniform time, early in the latter half of the instar.

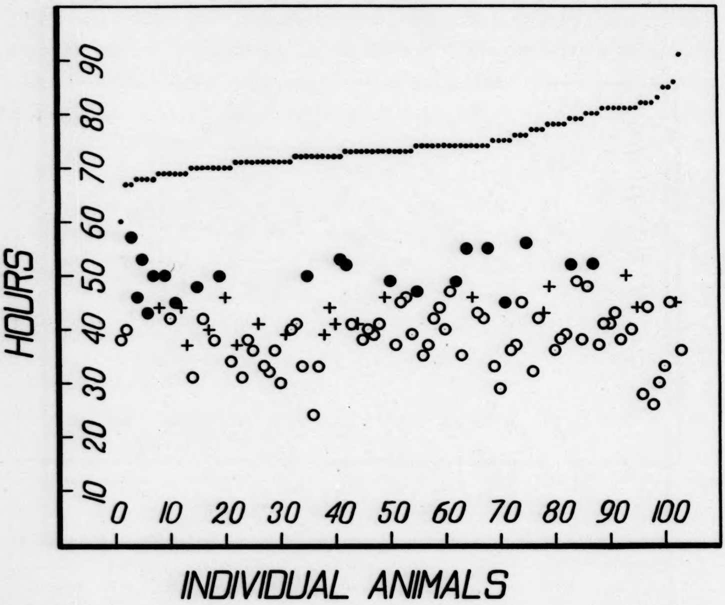


FIG. 3.—A graph showing instar lengths and operative hours for the experiment at 22° C. The base line corresponds to the beginning of the instar for each individual animal. The small solid circles represent instar lengths. The large solid circles represent times of operation which produced wounds resembling Figure 1, *C;* the crosses, Figure 1, *B;* and the open circles, Figure 1, *A*. The mean instar length was 74.22 ± 0.34 hours for the 103 operated animals and 73.74 ± 0.76 hours for the 27 unoperated controls. For animals displaying Class B wounds the instar length was 73.63 ± 0.84 for 19 cases. The mean operative time in per cent of individual instar lengths of the latter animals was 58.61 ± 0.78.

A comparison of the mean operative times from each experiment for the operations yielding Class B wounds shows that in no event is the difference between the results greater than four times the probable error of the difference, indicating that the temperature coefficients for the initial secretion of chitin within the instar and for the length of the instar are of the same magnitude. When a composite graph (Fig. 5) of the three experiments is made by factoring the results of the experiments at 22° C. and 25° C. to the one at 15° C., using mean instar lengths for factors, and on dividing the graph into quadrants, it is to be noted that, whether the individual has a shorter or longer instar length than the average, the times of the beginning of chitin secretion are in the same proportion to the individual instar lengths.

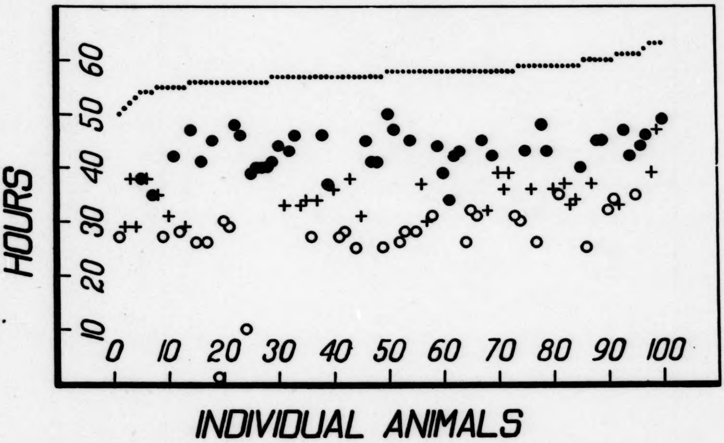


FIG. 4.—A graph showing instar lengths and operative hours for the experiment at 25° C. The base line corresponds to the beginning of the instar for each individual animal. The small solid circles represent instar lengths. The large solid circles represent times of operation which produced wounds resembling Figure 1, *C;* the crosses, Figure 1, *B;* and the open circles, Figure 1, *A*. The mean instar length was 57.51 ± 0.15 hours for the 100 operated animals and 57.06 ± 0.33 hours for the 53 unoperated controls. For animals displaying type B wounds the instar length was 57.38 ± 0.35 for 29 cases. The mean operative time in per cent of individual instar lengths of the latter animals was 60.89 ± 0.74.

These experiments show that regardless of the variation in instar

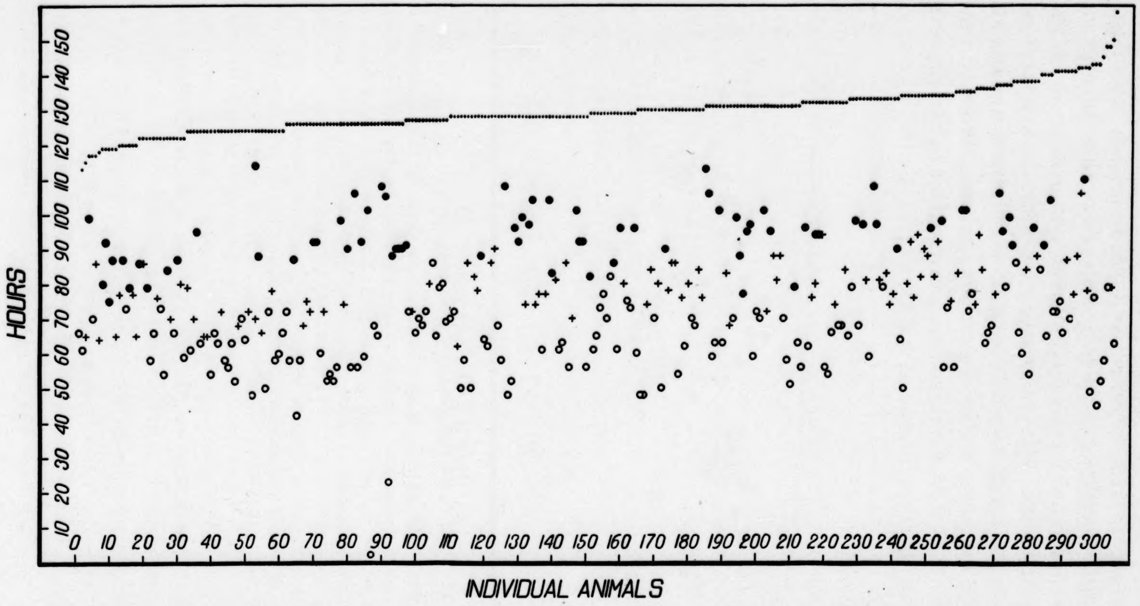


FIG. 5.—A composite graph made by factoring the data for Figures 1, 2, and 3 to Figure 1, using mean instar lengths as factors. The mean operative time in per cent of individual instar lengths for all type B wounds was 60.79 ± 0.42. Upon dividing the graph into quadrants, the operative times become for quadrant No. 1, 58.84 ± 0.75; for quadrant No. 2, 60.94 ± 0.93; for quadrant No. 3, 61.59 ± 0.67; for quadrant No. 4, 61.54 ± 0.70.

lengths due to different temperatures or other causes, under the conditions of the experiments, the formation of chitin for a given adult instar begins near the middle of the previous instar. Until nearly three-fourths of the instar has passed, the newly secreted layer is not of sufficient strength to carry over the brown material. Probably the hypodermal cells continue to secrete until well into the next instar, as shown by the close adherence of the chitin coat to the underlying cells during the first part of the period.

SUMMARY

1. An experimental method for the determination of the time at which chitin secretion in an organism (*Daphnia magna*) begins has been developed.
2. The time at which chitin secretion begins has been determined for adult instars of *Daphnia magna* for three temperatures and is approximately a period at which 60 per cent of the instar has passed.
3. For the temperature range studied (15°–25° C.) the times at which chitin secretion begins within the instar is a function of the temperature, and the temperature coefficient is of the same magnitude as that for instar lengths.

LITERATURE CITED

ANDREWS, E. A. 1906. Partial regeneration of the sperm receptacle in crayfish. Jour. Exp. Zoöl., III, 121.

BANTA, A. M. 1921. A convenient culture medium for daphnids. Science, N.S. LIII, 557.

HESSE, R., and F. DOFLEIN. 1910. Tierbau und Tierleben, I, 127–28. Berlin and Leipzig: Teubner.

JACKSON, H. 1890. IV. Studies in the morphology of the Lepidoptera. Part I. Trans. Linn. Soc. London. Second Ser., Vol. V, Zoölogy, pp. 143–86.

MACARTHUR, J. W., and W. H. T. BAILLIE. 1929. Metabolic activity and duration of life. I. Influence of temperature on longevity in *Daphnia magna*. Jour. Exp. Zoöl., LIII, 221.

POLYCYTHEMIA IN WHITE RATS[1]

L. B. NICE, MARY ELIZABETH MORRIS, AND W. P. ELHARDT
Department of Physiology, Ohio State University

THIS study was undertaken to determine the variations in the number of erythrocytes in the blood of white rats when given daily injections of adrenalin; when adrenalectomized and administered adrenalin daily; and also when subjected to excitement (anger and pain) in the normal, adrenalectomized, and splenectomized states.

Schneider and Havens (1915) with man; Lamson (1915, 1916, 1920) with dogs and cats; Binet, Cordot, and Fournier (1928) with dogs; and Izquierdo and Cannon (1928) with cats have shown that the injection of adrenalin into the blood stream causes an increase in the number of erythrocytes as measured by taking their counts shortly after the administration of the adrenalin. In our experiments, we wished to find whether this increased number of erythrocytes in the blood stream would be maintained for some hours after subcutaneous injections of adrenalin had been given.

In regard to emotional polycythemia Ferrari (1897) in man, Lamson (1915, 1916, 1920) in cats, Izquierdo and Cannon (1928) in cats, and Barcroft (1930) with the dog have shown that there is a decided increase in the number of erythrocytes in the blood after emotional excitement. We wished to test this condition on rats; first, in the normal state; second, when adrenalectomized; and third, when splenectomized.

In these experiments rats ranging from 100 to 150 days old were used.

EXPERIMENTAL PROCEDURE

Counts were made on normal rats showing the number of erythrocytes per cubic millimeter of blood drawn from the heart by means of a hypodermic syringe. Similar counts were made on these same animals after they had been given for some time daily injections of

---

[1] The results of the early experiments were presented to the Thirteenth International Congress of Physiology, at Boston (1929).

1 cc. of 1:25000 adrenalin chloride solution (Parke Davis & Co.). These injections were made subcutaneously into the groin so that the activity of the animal would massage the adrenalin out into the blood stream. Similar experiments were made on adrenalectomized rats.

A group of normal rats were subjected to excitement (anger and pain) by being strapped back downward to an animal-holder and teased from time to time for a period of 10 minutes with an interrupted Faradic current with a Harvard inductorium. Their blood counts were taken before and after this treatment. The second counts were taken about an hour after the first. Similar studies were made on adrenalectomized and splenectomized animals and also on a number which had been injected daily with adrenalin.

The blood was diluted with Hayem's solution in the ratio of 0.5 cc. to 100 cc., using the same mixing pipette; and duplicate counts were always made by the same individual in the same Max Levy counting-chamber. In the following tables the figures for each animal is the average of two similar counts.

On account of exciting the anger of these rodents, a condition necessary to avoid for the basal counts, we found it impractical to obtain a sufficient amount of blood for our purposes without an anesthetic.[1] We therefore decided to use ether because of its quick action and the rapid rate at which the rats would recover from its effect. It is known, however, that ether disturbs the blood of animals, especially when they show excitement in going under; yet we believe that for comparative purposes our results are not greatly influenced. Nevertheless, we do not claim that our counts may not to some extent be influenced by the ether anesthesia.

RESULTS

The effects of adrenalin.—There was an increase in the number of erythrocytes in the blood stream in normal rats subjected to daily injections of adrenalin in comparison to their counts before treatment. These effects are shown in Table I. The average number of erythrocytes in the eleven normal rats was 10,840,000, in comparison to 12,080,000 after they had been subjected to adrenalin. This is an increase of 11.4 per cent. This increase was found 18–20 hours after the last injection. The daily doses of adrenalin apparently kept the reservoir of erythrocytes in the body, or at least a portion of it out in the blood stream.

---

[1] We also needed a small amount of blood from a few of the animals for sugar determinations.

Similar effects to those shown in the normal rats were found with the adrenalectomized group. The average number of erythrocytes in eleven adrenalectomized rats was 9,480,000, in comparison to 10,490,000 in those injected with adrenalin. This is an average increase of 10.9 per cent.

TABLE I

ERYTHROCYTES IN NORMAL AND ADRENALECTOMIZED RATS AFTER THE INJECTION OF ADRENALIN

Normal Rats				Adrenalectomized Rats				
Sex	Before Injection	After Injection		Sex	Before Injection		After Injection	
	Millions in 1 Cu. Mm.	Days Injected	Millions in 1 Cu. Mm.		Days after Operation	Millions in 1 Cu. Mm.	Days Injected	Millions in 1 Cu. Mm.
♂.........	10.74	15	11.68	♂........	3	11.08	6	12.72
♂.........	11.71	10	12.37	♂........	2	10.47	10	12.00
♂.........	11.43	10	12.03	♂........	10	9.06	6	10.06
♂.........	11.29	14	13.23	♂........	10	9.41	6	10.01
♂.........	11.76	11	13.58	♂........	3	11.30	6	12.73
♂.........	10.91	11	12.37	♂........	12	9.07	8	10.25
♂.........	10.72	14	12.17	♂........	12	8.67	12	8.82
♂.........	10.97	10	11.28	♂........	14	8.54	4	9.41
♂.........	9.69	34	10.77	♂........	14	8.46	4	9.04
♂.........	9.20	15	10.89	♂........	10	9.02	6	10.06
♂.........	10.87	16	12.54	♂........	14	9.29	4	10.38
Av.....	10.84	....	12.08	Av....	....	9.48	....	10.49

The effect of excitement.—Normal rats subjected to Faradic stimulation gave an increase in the number of erythrocytes in every case, as is shown in Table II. This increase is more marked when the counts are made soon after stimulation in comparison to those done later. The average count before excitement was 10,960,000, in comparison to 12,640,000 in the same eight animals 3 minutes after excitement. This is an average increase of 15.3 per cent above the basal counts.

When similar counts were made on another set of eight rats after longer intervals after excitement—from 10 to 40 minutes—less change was shown; they averaged 9,540,000 erythrocytes before excitement and 10,650,000 afterward. This is an average increase of 11.6 per cent. The average counts for the two sets of sixteen rats were 10,220,000 before and 11,630,000 after excitement, giving an average increase of 13.8 per cent. These results are in accord with those of Izquierdo and Cannon on cats (1928).

In contrast to the normal series, the adrenalectomized rats showed no increase on the average in erythrocytes after excitement. The average count on six adrenalectomized rats before excitement amounted to 11,240,000, while those taken 3 minutes after excitement averaged 11,160,000, which is an average decrease of −0.7 per cent. On five adrenalectomized animals the average count before excitement was 10,000,000 in comparison with 9,890,000 from 10 to 60 minutes afterward. This is an average change of −1.1 per cent. The average of both adrenalectomized groups was 10,680,000 before and 10,580,000 after excitement, or −0.9 per cent. In these experiments a difference of a quarter of a million in the count was found to be of no importance.

In the splenectomized group the average number of erythrocytes before excitement in ten animals was 10,150,000 per cubic millimeter of blood, while after excitement it was 9,790,000, or −3.5 per cent.

The splenectomized group which was given adrenalin daily had an average for ten animals of 10,343,000 erythrocytes before excitement and 10,312,000 afterward, or −0.3 per cent. The results recorded in Table III show that there is no average increase in the blood count of splenectomized rats after excitement, whether or not they have been subjected to daily injections of adrenalin.

The groups of rats in Table III indicate that the increased erythrocyte counts in the previous experiments must have come from the splenic reservoir.

DISCUSSION

No attempt was made to measure the degree of excitement in these rodents. There was, however, a decided difference in the amount of anger shown by the normal and splenectomized rats in comparison to the adrenalectomized group. The latter on the whole

TABLE II

ERYTHROCYTES BEFORE AND AFTER EXCITEMENT IN NORMAL AND ADRENALECTOMIZED RATS

NORMAL RATS					ADRENALECTOMIZED RATS					
Sex	Before Excitement, Millions in 1 Cu. Mm.	After Excitement, Millions in 1 Cu. Mm.	Minutes after Excitement	Percentage of Increase	Sex	Days after Operation	Before Excitement, Millions in 1 Cu. Mm.	After Excitement, Millions in 1 Cu. Mm.	Minutes after Excitement	Percentage of Increase
Counts in Millions per Cubic Millimeter of Blood Drawn 3 Minutes after Excitement										
♂..........	10.28	12.17	3	18.4	♂..........	7	11.45	10.53	3	− 8.9
♂..........	11.41	12.22	3	7.0	♂..........	8	10.99	11.13	3	1.3
♂..........	11.46	12.44	3	8.6	♂..........	8	10.13	10.38	3	2.4
♂..........	11.02	11.91	3	8.0	♂..........	8	11.73	11.90	3	1.5
♂..........	10.96	13.18	3	20.0	♂..........	9	12.41	12.39	3	− 0.2
♂..........	10.94	12.56	3	14.7	♂..........	9	10.71	10.61	3	− 0.9
♂..........	10.52	13.89	3	31.9						
♂..........	11.51	12.80	3	11.2						
Av......	10.96	12.64	....	15.3	Av......	....	11.24	11.16	....	− 0.7
Counts in Millions per Cubic Millimeter of Blood Drawn 10–40 Minutes after Excitement										
♂..........	9.69	10.27	40	5.9	♂..........	3	10.02	9.05	60	− 9.6
♂..........	9.72	10.38	20	6.8	♂..........	2	9.72	9.74	15	− 0.2
		10.44	30	7.7	♂..........	3	10.73	10.08	10	− 6.5
♂..........	7.25	7.69	12	6.1	♀..........	2	11.30	11.32	17	− 0.2
♂..........	7.44	9.57	10	28.5	♀..........	3	8.24	9.24	10	12.1
♂..........	10.95	13.48	10	23.0						
♂..........	11.25	11.70	10	4.0						
♀..........	9.85	10.88	10	10.5						
		10.92	33	9.7						
♂..........	10.43	11.23	10	7.6						
Av......	9.54	10.65	....	11.6	Av......	....	10.00	9.89	....	− 1.1
Av. of all	10.22	11.63	....	13.8	Av. of all	....	10.68	10.58	....	− 0.9

TABLE III

Erythrocytes in splenectomized rats before and after excitement

Splenectomized Rats						Splenectomized Rats Injected with Adrenalin						
Sex	Days after Operation	Before Excitement, Millions in 1 Cu. Mm.	After Excitement, Millions in 1 Cu. Mm.	Minutes after Excitement	Percentage of Increase	Sex	Days after Operation	Days Injected	Before Excitement	After Excitement	Minutes after Excitement	Percentage of Increase
♂.....	78	11.14	10.65	5	−4.4	♂.....	71	46	10.39	10.22	3	−1.6
♂.....	58	7.07	7.31	5	3.3	♂.....	79	49	9.34	9.23	5	−0.8
♂.....	75	11.12	10.65	5	−4.2	♂.....	80	49	10.19	10.57	5	3.7
♂.....	72	11.79	10.88	8	−7.8	♂.....	80	49	10.33	11.07	8	7.1
♂.....	94	12.01	11.54	8	−3.9	♂.....	70	50	10.42	10.53	8	0.8
♂.....	90	10.61	10.55	10	−0.6	♂.....	70	50	10.92	10.82	8	−0.9
♂.....	97	10.76	10.57	10	−1.8	♂.....	70	50	9.96	9.65	10	−3.1
♂.....	58	10.63	10.54	40	−0.8	♂.....	57	51	10.22	10.50	10	2.7
♂.....	49	8.54	8.58	40	0.4	♂.....	71	46	10.94	10.41	75	−4.9
♂.....	52	8.51	9.47	30	9.4	♂.....	71	46	10.97	10.22	30	−6.8
Av..	....	10.15	9.79	....	−3.5	Av..	....	....	10.34	10.31	....	−0.3

exhibited less disturbance in response to Faradic stimulation than did the other groups.

The counts on the splenectomized rats were made on animals 49–97 days after operation. This precaution was taken to avoid the anemia period which may occur shortly after splenectomy.

After excitement we always observed a great increase in the number of small erythrocytes in the blood as compared to the basal counts in normal animals.

In comparing the basal counts in our normal animals with those of other workers, our rats average higher (see Adams and Shevket, 1929). We offer no explanation for this situation. Our animals originally came from the Wistar strain and are kept on a McCollum diet supplemented with fresh cabbage and occasionally dog biscuits and meat.

Hemoglobin determinations were made by the Sahli method on most of our rats; but because of the wide variations and lack of definite relation to the increased erythrocyte count in many cases, they are not included in our results.

In these experiments polycythemia occurred in normal rats after being injected with adrenalin and after excitement. A similar augmentation in the number of erythrocytes occurred in the adrenalectomized rats after being given daily injections of adrenalin, but failed to appear after excitement.

In the splenectomized rats polycythemia failed to appear after daily injections of adrenalin and after excitement (anger and pain).

Several workers have shown that small amounts of adrenalin cause the spleen to contract, viz., Strasser and Wolf (1905); Hoskins and Gunning (1917), Tournade and Chabrol (1924, 1925), and Borysiewicz (1928). Besides Barcroft and his co-workers (1923–30), Binet and his collaborators (1926–28) have shown that the spleen contracts under various conditions such as exercise, asphyxia, emotional excitement, and after death. Izquierdo (1928) has also shown that the spleen contracts in anoxemia.

Since polycythemia failed to appear in our animals after removal of the spleen, we are forced to the conclusion that the augmentation in erythrocytes came from the reservoir in this organ. Furthermore, since this condition did not appear after excitement in adrenalectomized rats with the spleen intact but did occur after the injections of adrenalin, it would appear that under certain conditions the stimulus for emptying the spleen comes from the adrenal glands.

SUMMARY

1. Daily injections of adrenalin chloride administered subcutaneously caused an increase in the number of erythrocytes in the blood of normal and adrenalectomized rats.
2. Excitement (anger and pain) in normal rats produced an augmentation in the number of erythrocytes in the blood.
3. Excitement in adrenalectomized rats produced no average increase in the number of erythrocytes.
4. Excitement in splenectomized rats produced no increase on the average in the number of erythrocytes.
5. The polycythemia came from the reservoir in the spleen.

LITERATURE CITED

Adams, A. E., and F. Shevket. 1929. Normal blood picture of white rats and changes in picture following thyroid feeding. Phys. Zoöl., II, 181–220.

Barcroft, J. 1930. Some effects of emotion on the volume of the spleen. Jour. Physiol., LXVIII, 375–82.

Barcroft, J., and H. Barcroft. 1923. Observations on the taking up of carbon monoxide by the haemoglobin in the spleen. *Ibid.*, LVIII, 138–44.

Barcroft, J., H. A. Harris, D. Orahovats, and R. Weiss. 1925. Physiology of spleen: a contribution to the physiology of the spleen. *Ibid.*, LX, 443–56.

Barcroft, J., C. D. Murray, D. Orahovats, J. Sands, and R. Weiss. 1925. Influence of spleen in carbon monoxide poisoning. *Ibid.*, pp. 79–84.

Barcroft, J., and L. T. Poole. 1927. The blood in the spleen pulp. *Ibid.*, LXIV, 23–29.

Barcroft, J., and J. G. Stephens. 1927. Observations upon the size of the spleen. *Ibid.*, pp. 1–22.

Binet, L. 1927. La physiologie de la rate. Paris: Achahine.

Binet, L., H. Cardot, and B. Fournier. 1928. Splénocontraction et polyglobulie par divers agents chimiques. Arch. Intern. de Physiol., XXX, 212–18.

Binet, L., H. Cardot, and R. Williamson. 1926. Le rôle de la rate dans la détermination de la polyglobule asphyxique. C. R. Soc. de Biol., XCV, 262–64.

Binet, L., and B. Fournier. 1926. La réaction splénique de la saignée. *Ibid.*, pp. 1141–42.

BINET, L., and R. WILLIAMSON. 1926. Recherches sur les variations du nombre des globules rouges suivant l'état de la fonction respiratoire. Skand. Arch. f. Physiol., XLIX, 92–93.

BORYSIEWICZ, A. 1928. Contraction de la rate provoquée par l'injection d'adrénaline et comportement du sang splénique chez les animaux. C. R. Soc. de Biol., XCIX, 931–34.

FERRARI, G. C. 1897. Ricerche globulimetriche negli stati emozionali. Riv. di Patol. Nerv. e Ment., II, 306–11.

HARGIS, E. H., and F. C. MANN. 1925. Plethysmographic study of changes in volume of spleen in intact animal. Amer. Jour. Physiol., LXXV, 180–200.

HOSKINS, R. G., and R. E. L. GUNNING. 1917. The effects of adrenin on the distribution of the blood. II. Volume changes and venous discharge in the spleen. *Ibid.*, XLIII, 298–303.

IZQUIERDO, J. J. 1928. Studies on conditions of activity in endocrine glands; polycythemia of acute anoxemia. *Ibid.*, LXXXVI, 145–59.

IZQUIERDO, J. J., and W. B. CANNON. 1928. Studies on conditions of activity in endocrine glands; emotional polycythemia. *Ibid.*, LXXXIV, 545–62.

LAMSON, P. D. 1915. The rôle of the liver in acute polycythaemia; a mechanism for the regulation of the red corpuscle content of the blood. Jour. Pharm. & Exp. Therap., VII, 169–224.

———. 1916. The rôle of the liver in acute polycythaemia. IV. Further observations on the effect of shutting off the arterial blood supply to the liver. *Ibid.*, IX, 129–54.

———. 1920. The part played by the liver in the regulation of blood volume. *Ibid.*, XVI, 125–34.

NICE, L. B. 1929. The number of erythrocytes in white rats under different experimental conditions. Amer. Jour. Physiol., XC, 461.

SCHNEIDER, E. C., and L. C. HAVENS. 1915. The changes in the content of haemoglobin and red corpuscles in the blood of man at high altitude. *Ibid.*, XXXVI, 380–97.

STRASSER, A., and H. WOLF. 1905. Über die Blutversorgung der Milz. Pflüger's Arch. f. d. g. Phys., CVIII, 590–626.

TOURNADE, A., and M. CHABROL. 1924. La contraction de la rate par excitation du splanchnique relève d'un double mécanisme nerveux et adrénalinique. C. R. Soc. de Biol., XC, 835–38.

———. 1925. Sécrétion réflexe d'adrénaline. XCII, 418–20.

INSECT DEVELOPMENT. I. FATTY ACIDS IN THE GRASSHOPPER EGG

(Four figures)

ELEANOR H. SLIFER
Zoölogical Laboratory, University of Pennsylvania

INTRODUCTION

DUE in no small part to Needham's excellent review of the literature up to 1925, interest in the chemical changes occurring during embryonic development has become pronounced in recent years. By far the greatest amount of work of this kind has been done with the hen's egg. Among cold-blooded forms, such eggs as those of the trout, salamander, rattlesnake, starfish, and sea urchin have received attention. Studies of the metabolism of insect eggs are much less common, and comparatively little is known concerning the changes in such foodstuffs as fat, for instance, during embryogenesis in this class of animals.

Tichomiroff (1885) gave analyses of the eggs of *Bombyx mori*, the silkworm moth. According to this investigator,

> Die Eier von B. mori entwickeln sich im Sommer nur bis zu einer gewissen Stufe und zwar bis zur Ausbildung der definitiven Keimblätter. In diesem Stadium ruhen die Eier den ganzen Winter. Bringt man sie aber z. B. im Februar in ein geheiztes Zimmer, ungefähr in die Temperatur von 18–20° R., so schlüpfen die Räupchen schon in zwei Wochen aus.

Tichomiroff brought eggs indoors in February, analyzed a portion at once and the remainder two weeks later when about to hatch. He found the hatching eggs to contain approximately one-half as much fat (ether-soluble extract) as eggs just brought inside. There was also a decrease in water, solids, protein, glycogen, and cholesterol. Lecithin and the nitrogenous bases rose in amount; and chitin, absent in the beginning, was present at hatching.

Farkas (1903) using the Liebermann-Székely saponification method, reported similar findings for fat in this same insect. Eggs ready to hatch contained 48.2 per cent less fat than when first brought indoors. Fat, he states, is not the only substance burned during the embryonic development of the silkworm, for of the total dry weight lost during incubation but 57.7 per cent could be accounted for as fat.

Six years later Weinland (1909) found that eggs of the flesh fly *Calliphora* contain 4.6 per cent of petroleum-ether extract and that this decreases as the embryo develops, but he gives no figures to show the extent of this loss. The egg of *Calliphora*, it may be noted, hatches about 1 day after it is laid.

Rudolfs, in 1926, analyzed the eggs of the tent caterpillar *Malacosoma americana* for ether-soluble materials and found these to decrease during development. He, however, analyzed entire egg masses, including their gelatinous covers, and did not work with a known number of eggs.

Dubois, in a two-page article published in 1893, described briefly an oil which he had extracted by means of alcohol and ether from the eggs of the Algerian locust, *Acridium peregrinum*. One kilogram of recently laid eggs yielded from 40 to 50 grams of this oil. He remarks:

> L'huile se modifie et disparaît dans l'œuf au fur et à mesure du développment. Elle sert vraisemblablement en grande partie à produire de la chaleur, car en plongeant un thermomètre dans une caisse d'œufs près d'éclore, j'ai trouvé une température oscillant entre 41° et 42° C.

As reported by Bodine (1925) the eggs of the grasshopper *Melanoplus differentialis* are particularly suitable for physiological studies. They can be obtained in large numbers, are of a good size, and can be separated readily from the material incasing them. Throughout development the respiratory quotients secured from these eggs lie, in nearly all cases, between 0.70 and 0.80 (Bodine, 1929). This suggests that fats are being used largely as a source of energy. Fiske and Boyden (1926) maintain that when uric acid is the principal end-product of protein metabolism, as it is in the chick, an R. Q. in the neighborhood of 0.70 does not necessarily indicate exclusive fat combustion. For example, they calculate that when egg-albumin is the sole source of protein and all the nitrogen is excreted in the form of uric acid, the respiratory quotient is 0.71. Since adult insects excrete uric acid, it is quite possible that the insect embryo may do so as well. In view of these results it was thought worth while to make a detailed, quantitative study of the fat content of the grasshopper egg from the time of laying until hatching.

In previous work of this character it has been customary, in practically every case, to extract the dried eggs for long periods with alcohol and ether and to term the substances so isolated "fats." Since other compounds besides fats are soluble in ether and alcohol, it was decided that the fatty-acid content would serve as a truer index of fat metabolism. In comparing the relative values of extraction and saponification methods in fat estimation, Leathes and Raper (1925) make the following statement regarding the introduction, late in the nineteenth century, of the latter method:

> In this way the common component of simple and complex fats alike, was determined; the component which gives to both their physiological significance, and which accounts for their high value in the economy of the organism.

MATERIAL AND METHODS

The eggs used in the series of experiments presented here were laid, in most cases, by wild grasshoppers (*Melanoplus differentialis*) caught outdoors in the late summer. The insects were confined in cages in the laboratory, as recommended by Carothers (1923) and Bodine (1925), and fed a varied diet consisting of mixed grasses, ragweed, lettuce, apples, etc. The bottom of each cage was filled to a depth of several inches with moist sand. This was sifted once a day, and the egg-pods obtained in this way were buried in clean, damp sand, in labeled containers, and placed at once in an incubator kept at 25° C. Every effort was made to keep the cages in a clean and sanitary condition and to have an abundant supply of fresh food available at all times. Eggs laid by grasshoppers raised in the laboratory were used in about one-fifth of the experiments. These were indistinguishable from eggs laid by wild females; and their water, solid, and fatty-acid content was found quite normal.

In analytic work, such as that recorded here, where the material under investigation is destroyed in each experiment, the necessity for using large numbers of eggs in order to eliminate individual variations is quite obvious. This has been emphasized by Needham (1925). Earlier workers—Farkas, Weinland, and others—have already pointed out the peculiar advantages possessed by small organisms in this respect. It is evident that 1,000 grasshopper eggs, weighing perhaps 3 grams, would be considerably easier to handle and analyze than 1,000 hen's eggs weighing something like 50,000 grams. On the other hand, it is extremely difficult to separate the insect egg into its various fractions—shell, yolk, embryo, membranes, etc. —and to analyze them individually as has been done with the hen's egg. But it is not altogether impossible. Tichomiroff (1885) and Farkas (1903) isolated the shells of silkworm-moth eggs and determined their composition.

In the present study the method described by Kumagawa and Suto (1908) was used, with some minor modifications, for the estimation of the higher fatty acids. Eggs of the desired age were removed from their pods and washed in several changes of distilled water until perfectly clean and free from every trace of sand or débris. A definite number of them—not less than 50 (from 0.125 to 0.350 grams of fresh material)—were then counted out onto a piece of filter paper. When part of the water had been absorbed, the eggs were transferred to a second piece of filter paper and kept in a warm place until they were quite dry and rolled readily on the paper. (This took from 5 to 10 minutes.) They were then weighed in a 150 cc. Pyrex beaker, the weight of which, together with a glass stirring-rod and a small piece of filter paper, had already been ascertained. The eggs were next crushed with the stirring-rod, using the small square of paper to prevent the loss of any material. This last was necessary because the shells of the eggs, except when recently laid, are under quite a tension and when broken the yolk may be ejected forcibly to a considerable distance.

The beaker with its contents was placed at once in a desiccator over $CaCl_2$, and the air partially exhausted. When 50 eggs were used, a constant weight was attained in something less than 24 hours.

After weighing, a mixture of 25 cc. of 25 per cent NaOH and 5 cc. of redistilled 95 per cent alcohol was added, a large watch glass placed over the mouth of the beaker, and the whole heated for an hour and a half in steam. It was found necessary to watch the mixture closely during the first 15 or 20 minutes. At this time the soaps formed tended to foam up, and if not watched rose above the rim of the beaker and were lost. Occasional stirring and careful regulation of the heat was sufficient to prevent this. After this preliminary period no further watching was required.

When saponification was complete, any material adherent to the watch glass was washed into the beaker with a small amount of hot water and the contents of the beaker poured quantitatively into a separatory funnel. The beaker was washed out repeatedly with boiling water, and the rinsings added to the liquids already in the funnel. After partial cooling, 25 cc. of concentrated HCl were added and the whole allowed to cool to room temperature. When quite cold, 40 cc. of redistilled ether were added and the funnel well shaken, care being taken to hold the stopper and stopcock firmly in place. After standing until separation was complete, the lower aqueous layer was drained off into a clean beaker. The ether, containing the fatty acids, was then run off into a second beaker, every precaution being taken to prevent the loss of the least trace of material. Twenty cubic centimeters more of ether were then placed in the funnel and a few cubic centimeters allowed to run through and rinse out the stem of the funnel, the rinsings being collected in the same beaker as were the first 40 cc. The watery solution was then returned to the funnel and reshaken with the ether remaining there. This second ether extract was then separated off and added to the first, and the whole process repeated a third time. The combined ether extracts were allowed to evaporate in a current of air or else spontaneously at room temperature. (This last took about 24 hours.) Drying was completed by careful warming under an electric light. When perfectly dry and while still warm, 15 cc. of redistilled petroleum ether were added. The beaker was covered and allowed to stand at least ½ hour—usually longer—and the petroleum-ether extract was filtered through ether-washed cotton into a tared beaker. The extraction was repeated four or more times, using 5–10 cc. of petroleum ether each time. The combined extracts were allowed to evaporate spontaneously until a constant weight was attained. This was done conveniently by allowing the extract to evaporate overnight in a dust-free place. The extract contained, in addition to fatty acids, any ether- and petroleum-ether-soluble substances (such as cholesterol) which may have been present. These can be separated from the acids and estimated, according to Leathes and Raper (1925), only when at least a gram of crude acids has been isolated. From 50 grasshopper eggs, from 0.006 to 0.020 gram may be obtained according to the size and state of development of the eggs.

In order to test the method a number of control analyses were made with pure oleic acid, using it in quantities comparable to the amounts of fatty acid secured from the grasshopper eggs. The average amount of acid recovered in ten experiments was a little less than 98.0 per cent of the quantity taken.

RESULTS AND DISCUSSION

It will be helpful, perhaps, to review, as briefly as possible, some facts concerning the behavior of these eggs as noted by Carothers (1923) and Bodine (1925, 1929). In the vicinity of Philadelphia *Melanoplus differentialis* lays its eggs in the late summer and fall. These remain in the ground over winter and hatch, as a rule, late in May. If the eggs, immediately after being laid, are placed in an incubator at 25° C., they will develop for about 3 weeks. Growth then stops rather abruptly and the eggs remain in this quiescent condition for several months. Carothers, in 1923, noted for several species of grasshoppers that "numerous embryos sectioned during this period showed scarcely a cell in division." In the present study cytological preparations made of *Melanoplus differentialis* embryos 20 days old were found to contain as many as 4,400 mitotic figures, while sections made of embryos a week older showed a complete, or an almost complete, absence of dividing cells.

In *Melanoplus differentialis*, in rare instances, hibernation fails to occur, a few embryos revolve around the posterior pole of the egg on the twenty-third or twenty-fourth day at 25° C.—sometimes this takes place a few days later—and hatching begins about the thirty-eighth day. Carothers, in her 1923 paper, also remarked the fact that occasionally, in a pod of otherwise hibernating eggs, a few grasshoppers will hatch without passing through a prolonged resting stage. In certain species, such as *Chortophaga viridifasciata*, development proceeds normally from beginning to end without a pause, and hatching occurs about the sixth week.

Melanoplus differentialis eggs in which growth had recommenced after an embryonic hibernation of 5 months were observed to hatch in approximately the same number of days after revolution as did the eggs which failed to hibernate. Revolution is easy to follow in such eggs, for the eyes are deeply pigmented and plainly visible through the semitransparent shell.

The curve for oxygen consumption for these eggs given by Bodine (1929) shows an early rise with a peak at the nineteenth day, or thereabouts, followed by a fairly sharp drop. By the thirty-fifth day at 25° C. the consumption of oxygen has reached a low level and remains there for a long period. About 2 weeks before hatching, the curve begins to rise again, and the rate increases steadily until the fully formed insect is ready to emerge. As do the other metabolic activities so far studied, the passage of water into the egg from the external environment also comes to a standstill at the onset of hibernation, to be resumed only when the embryo again begins to grow.

If eggs laid in the fall, instead of being kept at a high temperature, are put at once in outdoor cages and allowed to remain there until December or later, these eggs, when brought inside and placed at 25° C., will begin to develop rapidly and will hatch within a few weeks (Bodine, 1929). Exposure to low temperatures in some way breaks or prevents the occurrence of the embryonic diapause. The time required for hatching under these conditions, as might be expected, depends upon the degree of development when first brought indoors, and this, in turn, upon the temperature to which the egg has been exposed. Eggs laid early in the fall will be found in a more advanced state than eggs laid later.

The interpolation of a lengthy resting period into the developmental cycle is paralleled in the eggs of other orders of insects. It is not at all uncommon to find, as the normal state of affairs, eggs laid in the fall hatching the following spring. Whether such eggs would develop without interruption if kept at favorable, high temperatures from the time of laying is, in most cases, unknown. As was noted earlier, Tichomiroff (1885) described for the silkworm moth conditions comparable to those found in the grasshopper.

The present study deals with the fatty-acid content of (1) eggs kept at 25° C. from the day they were laid and (2) eggs first exposed to low outdoor temperatures and later placed at 25° C. until hatched. In the first case, the eggs were analyzed when 1 day old and at weekly intervals thereafter, counting from the time of laying, for seven weeks. One hundred and fifty-nine analyses representing a total of more than 20,000 eggs were used in the establishment of the curve covering the early period shown in Figure 1. In a single analysis, as a rule, the eggs in one pod (generally 100 or more in number) were used. Occasionally eggs from several pods were combined in a single experiment. Although there is considerable variation in the amount of fatty acid obtained from eggs from different pods, the differences are no greater than are the variations found in the size and weight of the eggs themselves. This becomes evident when results are expressed in terms of percentage composition. The eggs

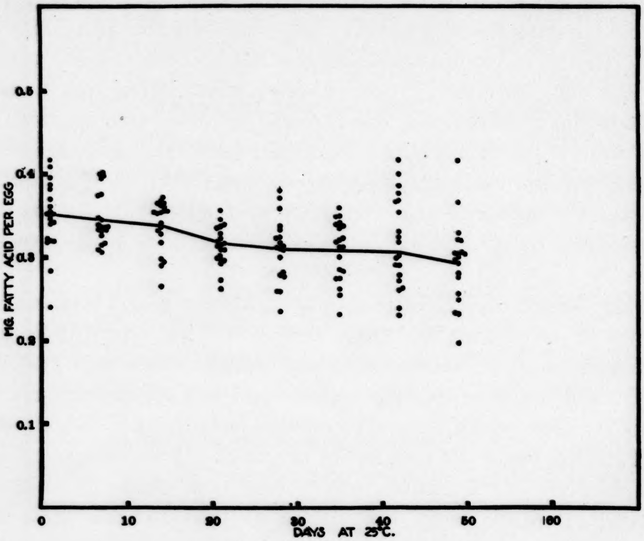


FIG. 1.—Fatty acids in *Melanoplus differentialis* eggs kept at 25° C. from day of laying. Analyses made at 1-day and at end of first, second, third, fourth, fifth, sixth, and seventh weeks. Each point represents a single analysis. Curve drawn through "weighted" averages for each week.

from any one pod are, however, remarkably uniform in their content of water, solids, and fatty acid.

The curve shown in Figure 1 is drawn through the "weighted" averages for each week which were obtained by multiplying the figure for the amount of fatty acid in each single analysis by the number of eggs used in that particular experiment, adding all the results and dividing by the total number of eggs used in all the experiments for that particular age.

Each newly laid *Melanoplus differentialis* egg contains, on an average, 0.352 mg. of fatty acid. This comprises from 9 to 12 per cent of the wet weight, or from 17 to 22 per cent of the dry weight. These results are to be compared with the data in Table I for fat in insect eggs given by previous workers. All represent the condition at or shortly after the time of laying, with the exception of the figures for *Bombyx mori*, which are based on eggs analyzed during the winter hibernation.

TABLE I

Investigator	Species	Fat in Egg (Percentage of Wet Weight)	Method
Tichomiroff, 1885	*Bombyx mori*	8.08	Ether extraction
Dubois, 1893	*Acridium peregrinum*	4.0–5.0	Ether and alcohol extraction
Farkas, 1903	*Bombyx mori*	7.34	Saponification
Weinland, 1909	*Calliphora*	4.60	Petroleum-ether extraction
Rudolfs, 1926	*Malacosoma americana*	9.0	Ether extraction

As development proceeds in the grasshopper egg, the amount of fat falls, slowly during the first 2 weeks, then more rapidly until the beginning of the fourth week, at which time each egg contains about 0.040 mg. less acid than when first laid. After the fourth week little change occurs. It is probable that the low average for the seventh week has no especial significance but is due rather to the unusual amount of variation encountered in the eggs chosen at this stage.

In order to study the later period of development, ten series of eggs kept outdoors from the time they were laid were brought inside, placed at 25° C., and samples analyzed, as a rule, every second day until hatching occurred. Instead of analyzing single pods, as was done in the earlier part of the work, a number of pods (25 or more) were opened at the beginning of each series and the contents thoroughly cleaned and mixed. Representative samples of 100–150 eggs were then selected from the lot at the desired intervals and analyzed. In this way average results for very large numbers of eggs were obtained. Curves so secured are shown in Figures 2 and 3. As the embryo increases in size, the fatty-acid content falls rapidly; and at hatching, less than half (on an average 45.7 per cent) of the amount found in freshly laid eggs remains.

Figure 4 is a synthetic graph made up of the first three "weighted" averages from Figure 1 and the entire curve for Series J from Figure 3. It will be remembered that occasionally *Melanoplus differentialis* eggs fail to hibernate when kept at 25° C. and hatch about the thirty-eighth day. Since the eggs in Series J began to hatch on the eighteenth day indoors, it may be assumed that when first brought inside they had reached a stage corresponding to the twentieth day of development at 25° C. This was verified by an examination of the embryos themselves, the majority and largest of which were within a day or so of this age. Similar composite curves can be prepared using for the latter portion any of the other series, the fit being very close with Series C, D, E, F, G, H, and K but less satisfactory with Series A and B.

During the present study a single analysis of the eggs of another member of the Acrididae, *Chortophaga viridifasciata*, was made. This grasshopper lays eggs in the spring which hatch in the summer and so are never exposed to cold weather. The fatty acids obtained from its eggs solidified, at room temperature, immediately after isolation. The acids from *Melanoplus differentialis*, on the other hand, which are subjected normally to very low temperatures, remain liquid for several days after isolation; crystallization then begins but progresses very slowly, a large proportion of the acids still being liquid a year later. The apparent suitability of the fatty acids in each of these cases for the particular conditions under which the egg develops seems worthy of further attention.

The shell of the freshly laid egg of *Melanoplus differentialis* is white or ivory-colored and disintegrates readily when treated with strong alkali. Shortly after laying, it becomes a uniform or mottled

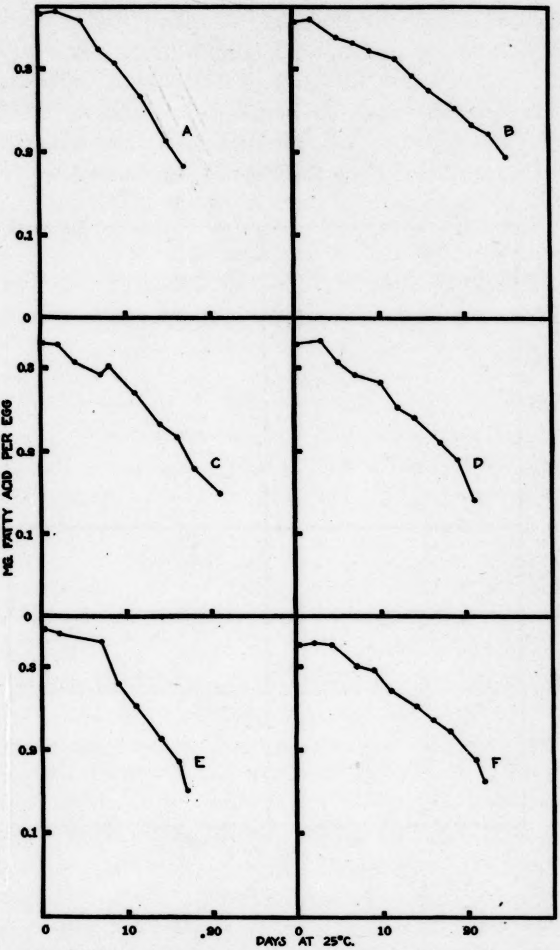


FIG. 2.—Fatty acids in *Melanoplus differentialis* eggs first exposed to low, outdoor temperatures and later placed at 25° C. until hatched.

A, eggs laid September 23, 1929; out to December 11; 25° C. to hatching.
B, eggs laid October 16, 1929; out to December 12; 25° C. to hatching.
C, eggs laid October 3, 1929; out to December 16; 25° C. to hatching.
D, eggs laid October 10, 1929; out to December 21; 25° C. to hatching.
E, eggs laid September 26, 1929; out to December 30; 25° C. to hatching.
F, eggs laid October 15, 1929; out to January 6, 1930; 25° C. to hatching.

yellowish-brown, and after the eighth to tenth day at 25° C. is no longer destroyed by prolonged boiling with 25 per cent NaOH. Campbell (1929) tested the eggs of *Melanoplus differentialis* for chitin and found that "the chorion does not contain chitin, but when the egg has developed to a point where the blastoderm cells have laid down a membrane upon the chorion, that membrane contains chitin."

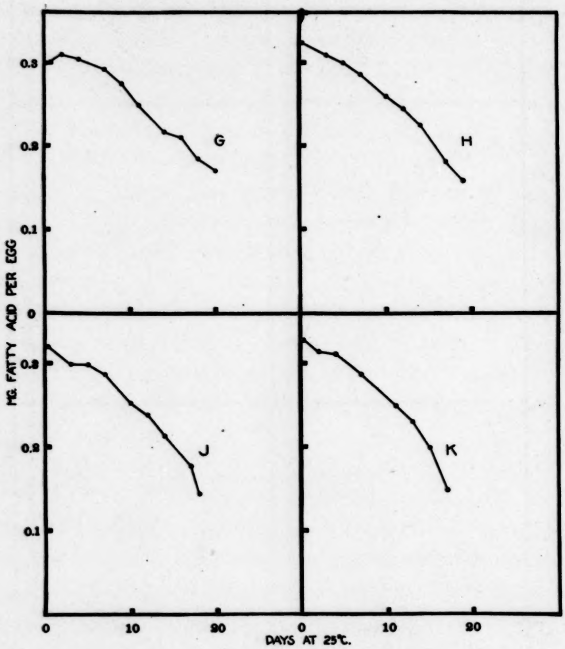


FIG. 3.—Same as Figure 2.
G, eggs laid October 4, 1929; out to January 7, 1930; 25° C. to hatching.
H, eggs laid October 11, 1929; out to January 11, 1930; 25° C. to hatching.
J, eggs laid October 3, 1929; out to January 17, 1930; 25° C. to hatching.
K, eggs laid September 23, 1929; out to January 28, 1930; 25° C. to hatching.

Shells secured from hatching animals were found to compose, in one experiment, about 12.5 per cent of the dry weight, or 3.0 per cent of the wet weight.

For *Bombyx mori* Tichomiroff (1885) reported a loss of water as the egg developed, the wet weight decreasing some 11.6 per cent. The greatest change occurred during the first 9 days after the eggs were brought inside; after this the loss was comparatively small. By the ninth day the dorsal wall of the embryo had grown so as to completely inclose the yolk, and Tichomiroff believed that this hindered the further loss of water. The behavior of the silkworm moth egg, in this respect, is the exact opposite of that of the grasshopper. Large quantities of water are taken up by the egg of *Melanoplus* *differentialis*, so that at hatching it weighs more than twice as much as when first laid (Bodine, 1929). Following revolution and the closure of the dorsal wall, the rate of water intake falls off decidedly. Perhaps Tichomiroff's argument may be applied here, the wall of the embryo now preventing the passage of water into, rather than out of, the egg.

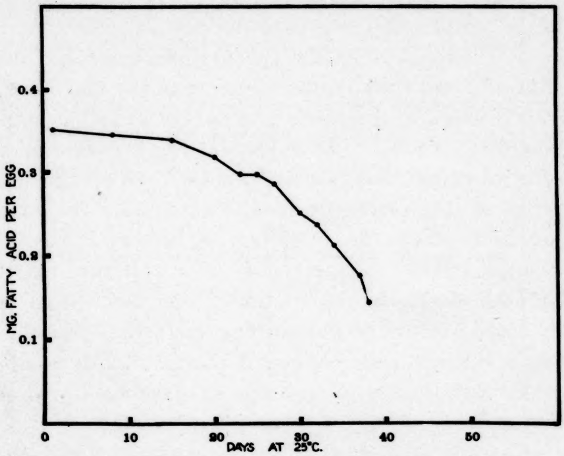


FIG. 4.—Fatty acids in *Melanoplus differentialis* eggs. "Synthetic" curve made up of first three averages from Figure 1 and curve J from Figure 3. Further explanation in text.

Dubois' (1893) description of the yolk of the recently laid egg of the Algerian locust fits equally well that of *Melanoplus*. "Soumis à la presse, les œufs ont laissé échapper le vitellus sous forme d'un fluide visqueux, épais, semblable à du miel." As development proceeds in the *Melanoplus differentialis* egg and the water content rises, the yolk becomes very much less viscous; and at the time when the embryo is passing around the caudal pole of the egg, is extremely thin and watery. Possibly this condition facilitates the passage of the embryo through the yolk.

Needham, both through his own work and through an elaborate survey of the literature on embryonic metabolism, has come to the conclusion that there are two types of eggs—aquatic and terrestrial. The eggs of fishes and Amphibia belong to the first group; and the eggs of birds, reptiles, and insects to the second. The latter cleidoic, or "closed-box" eggs as Needham (1929) terms them, are more completely shut off from their surroundings than the non-cleidoic; and in them the disposal of the waste products of metabolic activity is, in consequence, not easy. The terrestrial egg avoids this difficulty (1) by using fat rather than protein as a source of energy and (2) by excreting uric acid in place of urea and ammonia. The combustion of fat is advantageous to the closed-box egg because it leaves no non-volatile residue; indeed, the metabolic water so formed may even be a valuable addition to the egg contents. The waste from the comparatively small amount of protein burned in the terrestrial egg is excreted as uric acid. This, because it is only slightly soluble, does not diffuse through the egg and give rise to toxic conditions, as would urea or ammonia.

In so far as the grasshopper egg is terrestrial and burns large quantities of fat during development, its behavior lends additional support to Needham's view.

SUMMARY

1. The newly laid egg of the grasshopper *Melanoplus differentialis* contains, on an average, 0.352 mg. of the higher fatty acids. This comprises from 9 to 12 per cent of the total wet weight, or from 17 to 22 per cent of the total dry weight.

2. As development proceeds, the amount of fatty acid falls; and at hatching, less than 50 per cent of the amount originally present remains.

3. During the early stages of hibernation the amount of fatty acid present changes little.

4. The fatty acids isolated from the eggs of *Chortophaga viridifasciata* have a higher melting-point than the acids isolated from the eggs of *Melanoplus differentialis*. Possibly this may be correlated with the differences in temperature to which the eggs of these two species are normally subjected.

5. Chitin is deposited beneath the chorion of the *Melanoplus* egg in sufficient quantities between the eighth and tenth days at 25° C. to prevent complete disintegration of the egg after that time by boiling NaOH.

6. The rate of water intake falls off decidedly shortly after revolution, which fact is due, perhaps, to the closure of the dorsal wall of the embryo.

7. The decrease in viscosity of the egg contents caused by the passage of water into the egg may facilitate the movements of the embryo through the yolk during revolution.

8. The consumption of large quantities of fat by the developing grasshopper embryo furnishes new support to Needham's views concerning fundamental differences to be found between the metabolism of terrestrial and of aquatic eggs.

The author is particularly indebted for advice and encouragement to Dr. J. H. Bodine, under whose direction the present work was begun, and to Dr. L. V. Heilbrunn, with whom it was continued. Thanks are due also to Dr. D. Wright Wilson, of the Department of Physiological Chemistry, for criticism of the method employed; to Dr. C. E. McClung, and others of the Zoölogy Laboratory, who read the manuscript and made valuable suggestions during the course of the work; and to Dr. P. R. Orr, who helped many times in securing supplies and apparatus.

LITERATURE CITED

Bodine, J. H. 1925. I. Effect of temperature on rate of embryonic development of certain Orthoptera. Jour. Exp. Zoöl., XLII, 91.

———. 1929. Factors influencing the rate of respiratory metabolism of a developing egg (Orthoptera). Physiol. Zoöl., II, 459.

Campbell, F. L. 1929. The detection and estimation of insect chitin; and the irrelation of "chitinization" to hardness and pigmentation of the cuticula of the American cockroach, *Periplaneta americana* L. Ann. Ent. Soc. Amer., XXII, 401.

Carothers, E. E. 1923. Taxonomy, development and life-history of certain Acrididae (Orthoptera). Trans. Amer. Ent. Soc., XLIX, 7.

Dubois, R. 1893. Sur l'huile d'œufs de la sauterelle d'Algerie ou criquet pèlerin (*Acridium peregrinum*). Compt. rend. Acad. Sci., CXVI, 1393.

Farkas, K. 1903. Beiträge zur Energetik der Ontogenese. Dritte Mittheilung. Ueber den Energieumsatz des Seidenspinners während der Entwicklung im Ei und während der Metamorphose. Archiv. f. die gesammte Physiol., XCVIII, 490.

Fiske, C. H., and E. A. Boyden. 1926. Nitrogen metabolism in the chick embryo. Jour. Biol. Chem., LXX, 535.

Kumagawa, M., and K. Suto. 1908. Ein neues Verfahren zur quantitativen Bestimmung des Fettes und der unverseifbaren Substanzen in tiereschem Material nebst der Kritik einiger gebräuchlichen Methoden 1. Biochem. Zeitschr., VIII, 212.

Leathes, J. B., and H. S. Raper. 1925. The fats. London: Longmans, Green & Co.

Needham, J. 1925. The metabolism of the developing egg. Physiol. Rev., V, 1.

———. 1929. Protein metabolism and organic evolution. Sci. Progress, XCII, 633.

Rudolfs, W. 1926. Studies on chemical changes during the life cycle of the tent caterpillar (*Malacosoma americana* Fab.). I. Moisture and fat. Jour. New York Ent. Soc., XXXIV, 249.

Tichomiroff, A. 1885. Chemische Studien über die Entwicklung der Insecteneier. Zeitschr. f. physiol. Chemie., IX, 518.

Weinland, E. 1909. Chemische Beobachtungen an der Fliege Calliphora. Biol. Centralbl., XXIX, 564.

A QUANTITATIVE STUDY OF THE EFFECT OF CHLOROFORM ON OXYGEN CONSUMPTION OF THE TADPOLE (RANA CLAMITANS)

SIDNEY A. NARINS
Department of Biology, University College, New York University

INTRODUCTION

THE effect of anesthetics on the irritability and respiratory exchange of organisms has been widely studied. The material used includes animals and plants, and ranges in the former from Protozoa to man. Although the results have been contradictory in some instances, several have been obtained so repeatedly that it is safe to state them as phenomena which can generally be expected to occur. These results will be summarized briefly here: First, dilute solutions of depressing substances generally stimulate irritability and respiratory exchange. This effect is demonstrated by the work of the following men: Demoor (1894) on protoplasmic rotation in plant cells; Lee and Salant (1902), on the oxygen consumption of muscle tissue; Carlson (1906), on the effect of various depressing agents on the nerve cells controlling the heart beat of *Limulus;* Vernon (1912), on the oxygen consumption of isolated kidney tissue; Tashiro and Adams (1914), on the carbon-dioxide production in nerve tissue; Buchanan (1923), on the oxygen consumption of *Planaria;* and others. A second general conclusion is that more concentrated solutions of depressing agents, within certain limits, cause typical narcosis and markedly depress irritability and oxygen consumption. The work of Schroeder (1907), on *Aspergillus niger;* Batteli and Stern (1907*a*, 1907*b*), on minced tissues and organs of vertebrates; Hyman, on *Suberites*, a marine sponge (1916), on coelenterates and some annelids (1919*a*), and on Planaria (1919*b*); as well as that of Bodine (1922), on certain Orthoptera, are typical examples of this effect. Furthermore, it has been found that, when an animal is subjected to narcotizing concentrations of depressing agents over long periods of time, respiratory exchange drops to a low level during the early part of the exposure, at which point it remains fairly constant for as long a period as the anesthesia is complete. This phenomenon was found to apply to *Planaria* by Allen (1919), and Buchanan (1926), and to Orthoptera by Bodine (1923). Finally, recovery from anesthetizing concentrations is generally very rapid, subsequent to the removal of the anesthetizing substance. This is illustrated by the work of Vernon (1906), on isolated kidney tissue; Schroeder (1907), on *Aspergillus niger;* Onaka (1911), on red blood cells; Hyman (1919*a*) on coelenterates and some annelids; and Schanischewsky (1913), on a number of mammals.

It is surprising to find, from a survey of the literature, how little work has been done on amphibian larvae. The following two references are of interest, therefore, in this regard. Irwin (1918) tested the effect of ether on the carbon-dioxide production of tadpoles, frog eggs, *Fundulus* embryos, and the aquatic insect *Dineutes assimilis* (Aube). The concentrations used varied from 0.73 to 7.3 per cent. In every case the carbon-dioxide production was depressed, the degree of depression being dependent upon the concentration of ether used. Helff (1926) found it necessary to anesthetize *Rana pipiens* larvae with a 2 per cent solution of ether in order to keep them quiet during oxygen-consumption measurements. In measuring the oxygen consumption of animals so anesthetized, he found that there was an average increase in oxygen consumption of 19 per cent.

The present work concerns itself with a quantitative study of the effects of various concentrations of chloroform on frog larvae. The problem follows several main types of investigation and seeks to answer the following questions: First, what is the effect of narcotizing concentrations on oxygen consumption? Second, what is the effect of concentrations which do not cause complete anesthesia? Is there a stimulating concentration? Third, at what concentration is the threshold of effect noted? Finally, what is the effect of those intermediate concentrations between the highest reversible one and the threshold or lowest concentration to show a reversible effect?

The writer wishes to express his sincere gratitude to Dr. O. M. Helff, of this laboratory, for suggesting the problem, and for his friendly spirit and kindly interest during the course of the present work.

MATERIAL AND METHODS

The animals utilized in the present experiment were of the neotenous larval type of *Rana clamitans*. The specimens used were first-year forms, the eggs having been laid in the spring of 1929. They were sent to this laboratory from the vicinity of Cincinnati, Ohio, in shipments of 250 every two weeks during the months of December, 1929, to March, 1930. The tadpoles were well fed upon arrival and very well suited to the needs of the problem under investigation, as they could survive long periods of starvation. Individuals of the desired size were selected from each new shipment and kept until needed in several small aquaria.

The respiration of all individuals tested was based on Birge and Juday's (1922) modification of the Winkler idiometric method for measuring dissolved oxygen in water. A carefully standardized burette, graduated to the hundredth of a cubic centimeter, was used in titrating; and all titrations, whether by day or night, were read under diffused light of constant intensity. About five hundred Winkler tests were made in all.

The individuals selected for use in the tests ranged in body weight from 4 to 8.2 grams, but the majority of them weighed between 5 and 6 grams. The maximum body length was 95 mm., and the minimum 79 mm., while most of the specimens measured between 80 and 90 mm. The hind limb length varied from 8 to 22 mm., but the majority of specimens measured between 10 and 15 mm. Measurements were made merely as an indication of size, and no attempt was made to calculate the oxygen consumption per gram body weight. The tests were devised to determine the effect of various concentrations of chloroform on the animal as a whole, the comparison of metabolic rates of individuals of different sizes and ages not being under consideration.

The chloroform solutions used in the anesthetized tests were freshly prepared just before using, and were kept in rubber-stoppered flasks. Every precaution was taken to make these solutions as accurate as possible. A known amount of water was placed in a 500 cc. flask; and the desired amount of chloroform was carefully added to it, drop by drop, from a burette. The flask was then shaken thoroughly to insure complete solution of the chloroform in the water, and the whole was carefully diluted to 500 cc. The solutions were made up on a percentage of concentration basis. Since these solutions were used almost immediately after having been prepared, very little opportunity for volatilization was afforded. Undoubtedly, some volatilization did take place; but the percentage of error in concentration was about the same for each solution prepared, so that the comparative results obtained with their use were little affected.

The elimination of as many factors as possible which might influence, in any way, the oxygen consumption of the larvae during the tests was of great importance. First, as has been previously stated, the age and size of the individuals employed were maintained as constant as possible, considering the number of specimens tested. The second precaution consisted in the starvation of all specimens for at least 48 hours preceding the initial test. This removed any variation which might be due to peristaltic movements of the alimentary canal in the digestion of food. The temperature at which the tests were made was 25 ± 1° C.

The water used in the oxygen consumption tests was specially prepared a few days before it was needed, as follows: The container in which the test water was kept was filled about three-fourths full of tap water. Water was boiled for a few minutes to reduce the oxygen content below the desired level, and the boiling water was then added to the container. The container was corked, inverted several times to insure proper mixing, and allowed to stand for at least 48 hours. It was found that water prepared in this way gave satisfactory results, and that there was little variation in oxygen tension. Before the water was drawn to fill the flasks, the container was inverted several times to obtain uniform distribution of the contained oxygen throughout the volume of the testing-water.

Another obstacle was met, when it was found that tadpoles, taken from a 4-liter aquarium and placed in the confining quarters of a 500 cc. testing-flask, swam about rather vigorously at first. When they were left in the flasks for a period of several hours, however, this movement was greatly decreased, probably due to the fact that they had become somewhat acclimatized to their change of environment. The method of placing the tadpoles in the testing-flasks the night before they were to be used for the tests was tried, with marked success. After a period of 12 or more hours in the flask, the specimens had evidently become fully acclimatized, and there was consequently very little appreciable movement during the testing periods the next day. In a few cases it was found that considerable movement occurred during the tests, and data from such animals were consequently discarded. At the end of each testing period, after samples had been drawn, the remaining water was carefully poured out and fresh water admitted from the container. During this process tadpoles swam about; but by the time the flasks were closed off and replaced in the bath, they had once more settled to the bottom, and generally remained motionless during the succeeding test.

The experimental part of the present work was divided into three main series of experiments. Each of these series will be described and interpreted under separate headings.

RESULTS

SERIES I. NORMAL VARIATION IN OXYGEN CONSUMPTION

Preliminary tests showed that there was considerable hour-to-hour variation in oxygen consumption. It was therefore thought necessary to make a careful study of such variation, since this must be taken into consideration in evaluating data from experiments in which various concentrations of chloroform were used. Twenty-one animals were selected, ranging in body weight from 4.9 to 8.2 grams; and their oxygen consumption was determined, individually, for 4 successive hours.

The data covering this series of experiments are listed in Table I. It will be noted that the percentage of variation was not regularly calculated from the values of any two successive hours, but represented the greatest percentage of variation in oxygen consumption, i.e., the variation between the highest and the lowest hourly values, not considering the numerical order of the tests concerned. Although several instances were to be found of the greatest percentage of variation occurring between successive hours, as in animals No. 1, 3, 5, 10, and 19 (see Table I), it will be noticed that in animal No. 2 the percentage of variation was calculated from the values for hours 2 and 4; in animal No. 16, from hours 1 and 4; in animal No. 6, from hours 3 and 2; etc. As will be pointed out later, this manner of calculation of hourly variation for the normal series imposed a more severe test on the following series of experiments in which various concentrations of chloroform were used.

TABLE I

NORMAL VARIATION IN OXYGEN CONSUMPTION

ANIMAL NUMBER	BODY WEIGHT (GRAMS)	CUBIC CENTIMETERS OF OXYGEN CONSUMED BY WHOLE ANIMAL DURING CONSECUTIVE HOURS				GREATEST PERCENTAGE OF VARIATION
		First	Second	Third	Fourth	
1..........	8.2	0.5945	0.5797	0.6532	0.5510	7.3
2..........	7.6	0.7456	0.7494	0.6954	0.6559	12.4
3..........	7.1	0.4125	0.4870	0.4012	0.4210	17.7
4..........	7.0	0.4295	0.4462	0.4462	0.4596	6.5
5..........	6.7	0.3149	0.2626	0.2807	0.3089	18.7
6..........	6.3	0.3730	0.3282	0.3988	0.3497	17.6
7..........	5.1	0.2880	0.3732	0.3509	0.2435	34.0
8..........	5.9	0.3379	0.3150	0.2414	0.3172	28.0
9..........	5.7	0.2480	0.2381	0.2097	0.1857	25.0
10..........	4.9	0.5144	0.4018	0.4659	0.4703	21.0
11..........	5.2	0.4299	0.4876	0.4808	0.4600	11.0
12..........	4.9	0.5234	0.5642	0.4301	0.4280	24.0
13..........	5.8	0.5855	0.7289	0.7261	0.5931	19.0
14..........	6.6	0.5603	0.5722	0.4598	0.3924	31.0
15..........	6.2	0.4639	0.4291	0.4174	0.3238	30.0
16..........	7.2	0.6444	0.5596	0.4976	0.4421	31.0
17..........	7.0	0.6591	0.5157	0.4654	0.4031	38.0
18..........	7.3	0.6182	0.6747	0.6345	0.6917	10.6
19..........	6.3	0.5689	0.5806	0.4336	0.4875	25.3
20..........	5.6	0.5147	0.4367	0.4207	0.4435	18.2
21..........	7.9	0.3705	0.4402	0.3705	0.3348	23.9
Average.	....	0.4856	0.4844	0.4514	0.4268	21.4

Several general statements may be made from a survey of Table I. First, body weight was no indication of the amount of oxygen that would be consumed per hour or the degree of variation in oxygen consumption from hour to hour. Thus, in animals No. 4 and 17, although both weighed exactly the same amount, the latter showed a maximum percentage of variation almost six times as great as that of the former. In animals No. 9 and 13 the latter had an average hourly oxygen consumption about 66 per cent greater than the former, although their weight was practically the same. Again, in animals No. 1 and 12 the latter showed a maximum percentage of variation about three times that of the former, although it weighed slightly more than one-half as much as the other. Referring to animals No. 11 and 16, it will be noted that in this case the heavier animal displayed a maximum percentage of variation nearly three times that of the lighter one.

Another point apparent from Table I is that hourly oxygen consumption in a 4-hour testing period might vary in either direction. Although experimental conditions of oxygen tension, period of starvation, and temperature were maintained as uniform as possible, rises and falls in oxygen consumption, of rather unusual magnitude, were to be observed. The value for the second hour might be either higher or lower than that for the first hour; the third might vary in either direction in reference to the second; while the fourth hour showed the same unpredictable relationship to the third hour. Thus, in a 4-hour testing period, the hourly values might follow any of the possible permutations and combinations to give twenty-four different types of hourly fluctuations. Eight main types were found to occur in actual measurements of hourly oxygen consumption. It was, therefore, almost impossible to predict from the oxygen consumption during any one hour, what the consumption would be on the hour directly succeeding it. The fact that eight of the possible types of hourly fluctuations occurred, in addition to the fact that the variation in the average hourly oxygen consumption for the four hours tested was considerably less than the average greatest percentage of variation (see Table I), seemed to indicate that the present series was a true normal variation. The latter data have their significance in that the rises in oxygen consumption during any one hour, found in certain of the experimental animals, tend to be balanced by the depressions found in other animals tested during the same hour.

The greatest percentage of hourly variation varied from 6.5 per cent (No. 4) to 38 per cent (No. 17), while the average greatest percentage of variation was 21.4. This last figure is the most significant part of Table I. It should be reiterated here, that 21.4 per cent represents the average *greatest* percentage of *variation* (either up or down) between *any* two hours. It is thus seen that any concentration which can be said to have an effect must alter the oxygen consumption between hours, on the average, by more than 21.4 per cent. In later experiments in which various concentrations of chloroform were tested, the percentage of variation was always calculated from the values of a normal test and a *succeeding* anesthetized test. If the percentage of variation in the normal series was always calculated from the values of any one hour and the one directly succeeding it, it would be much smaller. Thus, any concentration which causes a variation or depression of oxygen consumption, as the case may be, between two succeeding hours, of more than 21.4 per cent, must be thought of as exerting a true effect.

SERIES II. EFFECT OF VARIOUS CONCENTRATIONS OF CHLOROFORM ON OXYGEN CONSUMPTION

In preliminary experiments it was found that freshly prepared solutions of chloroform, over 0.125 per cent in concentration, were lethal. A 0.5 per cent solution caused irreversible changes which were very rapid and vigorous. The body of an animal subjected to the latter concentration became contorted and discolored, and death always followed such an exposure. The concentrations between 0.5 per cent and the first concentration to show reversible anesthetic effects, i.e., 0.125 per cent, gave visible irreversible changes in keeping with their proportionate strength. A concentration of 0.01 per cent, on the other hand, was found to be incapable of producing complete anesthesia, no matter how long a tadpole was immersed in a solution of this strength. The action of this concentration, it was noticed, was to slow up body movement and response to mechanical stimuli during the first hour of the exposure, after which the animal seemed to recover from its effects. Similarly, external stimuli elicited a more marked response the second hour than was true of the first hour of exposure. Concentrations intermediate between 0.125 and 0.01 per cent produced complete anesthesia, lower concentrations requiring a longer time than higher ones. Ten tadpoles were used for each concentration in testing the time required to produce complete anesthesia.

It was thought of interest to test the effect of those concentrations of chloroform which showed reversible anesthetic effects. It was, furthermore, desired to notice what relationship existed between the concentration of chloroform employed and the effect observed. Another purpose of the present series was to investigate the recovery from various concentrations of chloroform. In this respect the tests in which 0.125 per cent chloroform was used were especially interesting. Preliminary experiments showed that the foregoing concentration caused complete anesthesia which lasted for 2 hours. Since it was to be expected from the results of previous workers that oxygen consumption would be depressed to a low level, at which point it would remain fairly constant for as long a period as the anesthesia was complete, the tests were devised to bring out this point. The concentrations of chloroform used in this series were: 0.125, 0.0625, 0.05, 0.04, 0.03, 0.02, and 0.01 per cent.

Thirty-six tadpoles were selected, varying in body weight from 3.7 to 7.8 grams, but a majority of them weighed between 4 and 5 grams. Eight tadpoles each were used for the tests with 0.01 and 0.125 per cent chloroform, and four tadpoles were used for each of the intermediate concentrations. The tests for the preceding two concentrations were run for 5 successive hours in the following way: A normal test was first run, which was followed by an anesthetized test in which the desired concentration of chloroform was used. This test was subsequently followed by three normal tests in successive order. Since 0.01 per cent chloroform was incapable of producing complete anesthesia, the anesthetized tests in this group were run in a solution of the foregoing concentration. In those tests in which the intermediate concentrations of chloroform were used, similar tests were run. Since the effect of and recovery from these concentrations were evident by the end of the fourth-hour test, a fifth-hour test was considered superfluous. The results of this series of experiments are presented in Table II.

It is evident from Table II that there is a direct relationship between the concentration used and the depressing effect observed. As was to be expected, higher concentrations of chloroform caused a greater absolute depression of oxygen consumption than lower ones, and a longer time was necessary for complete recovery from their effects. Lower concentrations, however, produced a greater proportional depression than higher ones. The tests in which the intermediate concentrations of chloroform were used illustrated the

TABLE II

EFFECT OF AND RECOVERY FROM VARIOUS CONCENTRATIONS OF CHLOROFORM

Animal Number	Weight (Grams)	Cubic Centimeters of Oxygen Consumed during Successive Hours (Whole Animal)											Percentage of Decrease First and Second Hours	Average Percentage of Decrease
		First Unanesthetized	Second Anesthetized							Third Unanesthetized	Fourth Unanesthetized	Fifth Unanesthetized		
			0.01%*	0.02%	0.03%	0.04%	0.05%	0.062%	0.125%					
1	6.0	0.5744	0.4360							0.5505	0.4986	0.5201	25	
2	6.7	0.5842	0.3102							0.4505	0.3838	0.3613	47	
3	7.8	0.4234	0.2798							0.3890	0.3308	0.3890	34	
4	4.3	0.6701	0.3965							0.5132	0.4247	0.4909	41	
5	4.5	0.5370	0.3595							0.6058	0.4683	0.4568	33	
6	5.1	0.6117	0.4701							0.5668	0.5325	0.6563	23	
7	4.4	0.5043	0.3821							0.5043	0.4380	0.5262	24	
8	6.1	0.5412	0.4296							0.5634	0.5193	0.5854	20	30.9
9	3.9	0.3406		0.1959						0.2798	0.3611		42	
10	4.6	0.6095		0.3471						0.5460	0.6019		43	
11	4.1	0.2717		0.1548						0.2445	0.2968		43	
12	4.3	0.1670		0.1052						0.1485	0.1670		37	41.3
13	4.1	0.2925			0.1504					0.2661	0.3331		48	
14	4.0	0.2860			0.1266					0.2224	0.2646		55	
15	4.3	0.3241			0.1943					0.2616	0.3648		40	
16	4.3	0.3099			0.1474					0.3318	0.2667		52	48.7
17	4.1	0.6248				0.2999				0.5639	0.6045		52	
18	4.5	0.4203				0.2505				0.3766	0.3344		41	
19	4.2	0.6098				0.2343				0.5576	0.6410		60	
20	5.8	0.4305				0.1701				0.3762	0.4081		60	53.2

* It was found that this concentration did not cause complete anesthesia. Measurements were, therefore, made in a solution of this strength.

TABLE II—*Continued*

Animal Number	Weight (Grams)	Cubic Centimeters of Oxygen Consumed during Successive Hours (Whole Animal)											Percentage of Decrease First and Second Hours	Average Percentage of Decrease
		First Unanesthetized	Second Anesthetized							Third Unanesthetized	Fourth Unanesthetized	Fifth Unanesthetized		
			0.01%*	0.02%	0.03%	0.04%	0.05%	0.062%	0.125%					
21	4.3	0.3785					0.2310			0.3154	0.3575		39	
22	4.4	0.2193					0.1097			0.2631	0.1976		50	
23	4.9	0.3792					0.1302			0.3585	0.3175		65	
24	4.7	0.4228					0.1334			0.3799	0.4441		68	55.5
25	5.5	0.4604						0.2139		0.4194	0.4831		51	
26	5.1	0.2716						0.1106		0.3571	0.2501		59	
27	4.8	0.4189						0.1679		0.3555	0.3136		60	
28	3.7	0.3036						0.1323		0.2420	0.2859		56	56.5
29	4.9	0.3466							0.0799	0.1006	0.3055	0.3669	80	
30	3.9	0.5671							0.1233	0.1475	0.4370	0.4770	79	
31	4.0	0.3917							0.1868	0.2081	0.3977	0.3555	53	
32	5.2	0.5681							0.1925	0.2066	0.4805	0.5469	66	
33	5.5	0.6178							0.1770	0.1974	0.5461	0.6075	71	
34	5.7	0.6478							0.2189	0.2322	0.6610	0.6265	66	
35	5.2	0.5228							0.2464	0.2910	0.4597	0.5440	55	
36	5.6	0.5359							0.1851	0.2069	0.5138	0.4918	70	67.5

foregoing generalization. With each progressive rise in concentration, the depressing effect on oxygen consumption became absolutely larger but proportionately smaller.

It was noticed that recovery from any concentration of chloroform (as indicated by a rise in oxygen consumption to a point within the normal variation) was complete within 1 hour after respiratory movements (the first visible sign of recovery) were resumed. Thus, if a concentration of chloroform caused complete anesthesia for 1 hour or less, oxygen consumption was normal by the end of the second hour; if it caused anesthesia which was complete for 2 hours, recovery was complete within 3 hours. It will be noted that 0.0625 per cent chloroform produced anesthesia which was complete for 1 hour, and animals so subjected showed a normal rate of oxygen consumption by the end of the succeeding hour. The remaining intermediate concentrations produced anesthesia which was complete for less than 1 hour, higher concentrations producing complete anesthesia for a longer period of time than lower ones. The exact time for recovery in the foregoing cases cannot be definitely stated in the present series, as the testing periods were always 1 hour in length. The probabilities are, however, that recovery was complete toward the beginning of the third hour, in a 4-hour testing period, if the concentration used produced complete anesthesia for only part of the second, anesthetized hour.

Finally, it will be noted that, from the data obtained when using 0.125 per cent chloroform, complete anesthesia caused a marked depression of oxygen consumption to a low point, where it remained constant for as long a period as the anesthesia was complete. Thus, it is seen in Table II that the oxygen consumption for hours 2 and 3, during which the anesthesia was complete, shows very little variation.

SERIES III. THRESHOLD CONCENTRATION IN RELATION TO OXYGEN CONSUMPTION

This final series of experiments concerned itself with an investigation of the effects of various concentrations of chloroform more dilute than 0.01 per cent and the presence or absence of a concentration which would cause a stimulation of oxygen consumption. Since a concentration as dilute as 0.01 per cent produced a depression of oxygen consumption which was considerable, despite the fact that it was incapable of producing complete anesthesia, the nature of the effects (if any were present) of concentrations more dilute than this one were sought. It was desired to find at what point the threshold of effect with chloroform lay, and whether this first effect was in the nature of a stimulation or depression. The concentrations of chloroform employed in the present series were 0.005, 0.00625, 0.0075, and 0.00825 per cent.

Twenty-one tadpoles were selected for use in the tests. These ranged in body weight from 4.2 to 8.2 grams, but the majority weighed between 4.5 and 6.5 grams. Four tadpoles were used for each of the first two concentrations, since the results were immediately evident; and eight and five, respectively, were used for each of the last two concentrations. The tests were run exactly like those in which the effect of 0.01 per cent chloroform was determined. These concentrations, too, were incapable of producing complete anesthesia, and the anesthetized tests were made in a solution of the concentration of chloroform being investigated. Since it was necessary to prepare sufficient solution to fill four testing flasks, these concentrations of chloroform were made up in 4-liter quantities. When the first normal test of the present series had been run, and test samples of water drawn off, chloroform was carefully added from a burette to a 4-liter container of water in sufficient quantity to make up the desired concentration. The container was then thoroughly shaken to insure complete solution of the chloroform in the water. This chloroform solution was immediately siphoned into testing-flasks, and the tests made as previously described. The results of this series are given in Table III.

The first concentration tested was exactly half 0.01 per cent, or 0.005 per cent, chloroform. When four animals had been tested, it became apparent that no effect was present. The variations in hourly oxygen consumption for the four hours tested were of the same general type as those obtained in Series I, in which four normal tests had been run. Two specimens showed a slight rise in oxygen consumption the second hour, while the other two showed a slight depression. Since these fluctuations were all well within the average for the normal series, it was concluded that this concentration was below the threshold point of effect of chloroform. The percentage of variation ranged from 2 to 16 per cent, the average being 6 per cent. The next higher concentration investigated was 0.00625 per cent chloroform. The testing of four tadpoles with this concentration served to show that the preceding statements applied here also, and therefore no further tests were run. The percentage of variation for 0.00625 per cent chloroform ranged from 2 to 15 per cent, the average variation being 5.5 per cent.

TABLE III

THRESHOLD CONCENTRATION IN RELATION TO OXYGEN CONSUMPTION

Animal Number	Weight (Grams)	Cubic Centimeters of Oxygen Consumed during Successive Hours							Percentage of Variation First to Second Hours	Average Percentage of Variation
		First Unanesthetized	Second Anesthetized*				Third Unanesthetized	Fourth Unanesthetized		
			0.005%	0.00625%	0.0075%	0.00825%				
1........	6.6	0.5535	0.5429				0.4909	0.5743	2	
2........	8.2	0.7134	0.5956				0.6349	0.6239	16	
3........	5.5	0.5018	0.5234				0.5129	0.4803	4	
4........	6.7	0.4163	0.4263				0.3937	0.4471	2	6
5........	4.5	0.5237		0.5060			0.4278	0.5926	3	
6........	4.8	0.3893		0.3815			0.4208	0.4756	2	
7........	6.1	0.5382		0.5267			0.4957	0.5592	2	
8........	5.4	0.6190		0.5116			0.4428	0.5312	15	5.5
9........	5.1	0.4635			0.5896		0.4076	0.4857	21	
10........	6.3	0.6025			0.7502		0.4770	0.5402	19	
11........	6.2	0.4807			0.5962		0.4770	0.4248	19	
12........	5.5	0.5414			0.6580		0.5195	0.4097	17	
13........	4.7	0.6354			0.5462		0.5462	0.5907	14	
14........	5.1	0.5452			0.6001		0.6523	0.5593	9	
15........	4.4	0.6755			0.5722		0.5974	0.6143	15	
16........	5.3	0.6297			0.5482		0.5983	0.6610	13	15.9
17........	6.1	0.7202				0.6105	0.6784	0.6994	15	
18........	4.8	0.5230				0.4316	0.4112	0.4338	17	
19........	5.2	0.5788				0.4486	0.4460	0.4905	22	
20........	6.3	0.7509				0.6465	0.5132	0.6838	14	
21........	4.2	0.5917				0.5504	0.4878	0.6180	7	15.0

* Since these concentrations did not produce complete anesthesia, anesthetized tests were made in a solution of the indicated concentrations.

The next concentration to be tested was 0.0075 per cent chloroform. The first four animals tested with this concentration all showed a rise in oxygen consumption for the second-hour test, which led to the conclusion that perhaps this was the stimulating concentration sought. Further tests, however, showed that the foregoing conclusion was somewhat premature, as three of the following four animals tested with this concentration showed a depression of oxygen consumption for the second-hour test almost as great in magnitude as the previously observed rises had been. In addition to this, every variation in oxygen consumption for the second-hour test was within the normal variation of 21.4 per cent (although animal No. 9 showed a rise almost as great). It was therefore concluded that this concentration, too, showed no effect; the data really represented normal variation in oxygen consumption.

The final concentration of chloroform tested in the present series was 0.00825 per cent. The variation in oxygen consumption for all five animals tested was of the same type, i.e., a drop the second, anesthetized hour, and normal variation the third and fourth hours. Although this suggested a possible depressing effect, it was found that the depression of oxygen consumption in every case (except animal No. 19) was within the average for the normal variation. Since the average percentage depression for the group, however, was within 21.4 per cent, this single exception lost its significance.

It was, therefore, concluded that none of the concentrations tested showed any measurable effect. No stimulating concentration was found, and the threshold point of depressing effect with chloroform must lie somewhere between 0.00825 per cent chloroform, which shows a depression within the normal variation, and 0.01 per cent chloroform, which shows the first true depressing effect on oxygen consumption.

DISCUSSION

It might seem unusual, at first, that as large a normal variation in oxygen consumption from hour to hour as has been shown to occur is present. It is difficult to understand why the same animal, tested with the same experimental precautions, shows, as in one case, a 38 per cent greater oxygen consumption one hour than it does during a preceding hour. It was at first thought that perhaps so large a normal variation was indigenous to a larval form such as the tadpole, but this is definitely not the case. Reference to the paper by Helff (1928) will show that as large, and in some cases even larger, hourly fluctuations in oxygen consumption are present in the normal adult crayfish. The explanation of this phenomenon is not obvious, to say the least. Helff, in the foregoing paper, offers several suggestions to explain normal variation, which will be given here. He states,

> It seems most likely that some system whereby a reserve supply of oxygen is maintained, exists . . . . when this reserve reaches a low point, as it is used up by the tissues, the external respiration of the animal then increases to build the reserve up to the maximum. In this way a steady supply of oxygen would be distributed to the tissues. . . . . A more logical and simpler explanation might be, that the individual cells of the animal are capable, in themselves, of storing surplus oxygen, which is then utilized at a certain rate, in connection with their various metabolic activities, until the supply is depleted. The reserve supply could then be thought of as being re-established. In this way, the oxygen tension of the blood would be subject to variation, and the attempt on the part of the animal to maintain it at a constant level, would result in a variation of the amount of oxygen consumed. . . . . The problem is an interesting one, however, and warrants investigation.

The present work agrees in the main with the results of previous workers, as stated in the introduction to this paper. Thus, it is found that within certain limits solutions of chloroform produce typical narcosis and a marked depression of oxygen consumption; complete recovery from deep anesthesia is found to follow rapidly the removal of the anesthetizing substance; and animals subjected to anesthetizing concentrations of chloroform maintain a uniformly low rate of oxygen consumption for as long a period as the anesthesia is complete. It differs in one respect from the general results of previous workers, in that no dilute concentration of chloroform was found which stimulated the oxygen consumption. Though a general survey of the literature would indicate that this result is unusual, a closer scrutiny will serve to show that other such cases are to be found—to wit, Hyman (1919*b*) and Allen (1919), working on *Planaria;* and Irwin (1918), who found no stimulating concentration of ether for frog tadpoles, frog eggs, *Fundulus* embryos, and aquatic insects. It should further be pointed out that chloroform shows an unusual depressing effect on the oxygen consumption of tadpoles. A 0.01 per cent solution was found to have an average depressing effect on oxygen consumption of 30.9 per cent, and this solution is almost exactly twice as dilute as that which Buchanan (1923) found produced a stimulation of oxygen consumption in cut pieces of *Planaria*.

In Series III it was noticed that several specimens tested with 0.0075 per cent chloroform showed an increase in oxygen consumption during the anesthetized hour; and in tests with a 0.00825 per cent solution, all specimens tested exhibited a decreased oxygen consumption during the anesthetized hour. Since it was found that these variations were all within the normal variation, it was concluded that neither of these concentrations could be said to have an effect. The probabilities are, however, that the first effect with chloroform on oxygen consumption, whether a stimulation or a depression, is a small one. Since the average normal variation in the present experiment, as has been pointed out, is comparatively large, any such effect (if any is present) would be obscured. It was thought, therefore, that if in some way the normal variation could be reduced to a minimum, a possible stimulating effect of chloroform might become apparent. An effort was therefore made to reduce the normal variation. As has been previously stated, an anesthetizing concentration of a narcotic substance showing reversible effects generally depresses the oxygen consumption to a constant low level for as long a period as the anesthesia is complete. Consequently, it was thought that if an animal was subjected to deep ether anesthesia for several successive hours, its rate of oxygen consumption would show very little variation. If this could be accomplished, then the concentration of chloroform to be tested could be superimposed on the deep ether anesthesia, and any effect present might become apparent. The tests, as run, were found to be unreliable. Despite the precautions taken, volatilization of the ether was very rapid. This made the depth of anesthesia during successive hours variable, and consequently the oxygen consumption on successive hours showed considerable variation.

The fact that it was impossible to reduce the normal variation in oxygen consumption makes it necessary to conclude that in the present work none of the concentrations of chloroform tested gave evidence of a stimulating effect and that the threshold concentration lies between 0.00825 and 0.01 per cent chloroform. The possibility exists, however, that a stimulating concentration is present and that the threshold concentration of chloroform is more dilute than that stated above, the magnitude of the normal variation obscuring these effects.

SUMMARY AND CONCLUSIONS

1. Twenty-one tadpoles (*Rana clamitans*) were tested for 4 consecutive hours, using a modified Winkler method for determining oxygen consumption. The hourly variation was found, in general, to be considerable, despite the fact that the same experimental precautions were observed in all tests. This normal variation must be considered in an evaluation of the results of experiments in which various concentrations of chloroform were used. The average greatest percentage of variation for the normal series was 21.4 per cent.

2. Thirty-six tadpoles were subjected to various concentrations of chloroform subsequent to normal tests, in investigating the effect of these concentrations on oxygen consumption. The percentage of concentrations used was: 0.01, 0.02, 0.03, 0.04, 0.05, 0.0625, and 0.125. All of the foregoing concentrations had a depressing effect. The average percentage of depressions was, respectively, 30.9, 41.3, 48.7, 53.2, 55.5, 56.5, and 67.5. Recovery from anesthesia is very rapid, a normal oxygen consumption being attained within 1 hour after respiratory movements are resumed. During deep anesthesia, the oxygen consumption is depressed to a low level, at which point it remains fairly constant for as long a period as the anesthesia is complete.

3. The effect on oxygen consumption of various solutions of chloroform whose concentrations were less than the lowest showing a reversible depressing effect was tested as above on twenty-one tadpoles. The percentage of concentrations used was 0.005, 0.00625, 0.0075, and 0.00825. The average percentage of variations in oxygen consumption produced was, respectively, 6, 5.5, 15.9, and 15. Since all of these variations were within the average greatest percentage of variation for the normal series, it was concluded that none of the concentrations tested showed any measurable effect. No concentration was found which stimulated oxygen consumption. The threshold point, it was concluded, lies somewhere between 0.00825 and 0.01 per cent chloroform.

4. An effort was made to reduce the normal variation by subjecting animals to deep ether anesthesia. The test, as run, was found to be unreliable, and it was found impossible to so reduce variation.

LITERATURE CITED

ALLEN, G. D. 1919. Quantitative studies on respiratory metabolism in *Planaria*. I. The effect of potassium cyanide on the rate of oxygen consumption. Amer. Jour. Physiol., XLVIII, 93.

BATTELI, F., and L. STERN. 1907*a*. Action de quelques substances sur l'activité respiratoire des tissus isolés. Jour. de Physiol., IX, 228.

———. 1907*b*. Recherches sur la conservation de l'activité de respiration dans les différents tissus d'animaux après la mort. *Ibid*., p. 410.

BIRGE, E. A., and C. JUDAY. 1911. The inland lakes of Wisconsin. The dissolved gases of the water and their biological significance. Wis. Geol. and Nat. Hist. Survey Bull., No. 22.

BODINE, J. H. 1922. Anaesthetics and carbon dioxide production. I. The effects of anaesthetics and other substances on the production of carbon dioxide by certain Orthoptera. Jour. Exp. Zoöl., XXXV, 323.

———. 1923. Anaesthetics and carbon dioxide production. II. Decreased carbon dioxide output and recovery from ether in certain Orthoptera. *Ibid*., XXXVIII, 413.

BUCHANAN, J. W. 1923. Weight changes and oxygen consumption during long exposures to dilute anaesthetics. *Ibid*., p. 331.

———. 1926. Some antagonistic and additive effects of anaesthetics and potassium cyanide. *Ibid*., XLIV, 285.

CARLSON, A. J. 1906. On the point of action of drugs on the heart with special reference to the heart of *Limulus*. Amer. Jour. Physiol., XVII, 182.

DEMOOR, J. 1894. Cf. F. Czapek, Biochemie der Pflanzen (Jena), I, 161.

HELFF, O. M. 1926. Studies on amphibian metamorphosis. II. Oxygen consumption of tadpoles undergoing precocious metamorphosis following treatment with thyroid and di-iodotyrosine. Jour. Exp. Zoöl., XLV, 69.

———. 1928. The respiratory regulation of the crayfish, *Cambarus immunis* (Hagen). Physiol. Zoöl., I, 92.

HYMAN, L. H. 1916. On the action of certain substances on oxygen consumption. I. The action of potassium cyanide. Amer. Jour. Physiol., XL, 238.

———. 1919*a*. The action of potassium cyanide on the oxygen consumption of coelenterates and some annelids. Biol. Bull., XXXVII, 404.

———. 1919*b*. On the action of certain substances on oxygen consumption. II. The action of potassium cyanide on *Planaria*. Amer. Jour. Physiol., XLVIII, 371.

IRWIN, M. 1918. Comparative studies on respiration. V. The effect of ether on carbon dioxide production by animals. Jour. Gen. Physiol., I, 209.

LEE, F. S., and W. SALANT. 1902. The action of alcohol on muscle. Amer. Jour. Physiol., VIII, 61.

ONAKA, M. 1911. Über die Wirkung des Arsens auf die roten Blutzellen. Zeitschr. f. Physiol. Chem., LXX, 433.

SCHANISCHEWSKY, A. 1913. Der Einfluss der Nitrile auf die Oxydationsprozesse und den Gasstoffwechsel des Organismus. Zentral. f. Biochem. u. Bio-Physik., XV, 780.

SCHROEDER, H. 1907. Über den Einfluss des Cyankalium auf die Atmung von *Aspergillus niger* nebst Bemerkungen über die Mechanik der Blausäurewirkung. Jahrb. f. Wiss. Bot., XLIV, 409.

TASHIRO, S., and H. S. ADAMS. 1914. Studies on narcosis. I. The effect of ethyl urethane and chloral hydrate on the carbon dioxide production of nerve fibre. Internat. Zeitschr. f. Phys. Chem. Biol., I, 450.

VERNON, H. M. 1906. The conditions of tissue respiration. Jour. Physiol., XXXV, 53.

———. 1912. The function of lipoids in tissue respiration and in the activity of oxidases. *Ibid.*, XVII, 182.

MITOTIC DEVELOPMENT OF ARBACIA EGGS IN CYANIDES

(One plate and five figures)

REUBEN BLUMENTHAL
Zoölogical Laboratory, University of Pennsylvania, and Marine Biological Laboratory, Woods Hole, Massachusetts

INTRODUCTION

MANY workers have pointed out that developing eggs of marine animals show a differential susceptibility to toxic agents and to hypotonic and hypertonic sea water during their cleavage cycle. Starting with the experiments of Lyon (1902), it has been shown that fertilized *Arbacia* eggs differ in their resistance to toxic agents, the degree of resistance varying according to the time after fertilization when the eggs were treated. This rhythm of susceptibility is repeated during each cleavage cycle. Lyon (1902), working with KCN and lack of oxygen, concluded that "from fertilization to a point ten or fifteen minutes after fertilization the resistance of the egg to KCN diminishes up to the time of first cleavage." In working with heat and cold, Lyon (1904) showed that *Arbacia* eggs are especially sensitive to heat just before cleavage. They are most resistant 10–20 minutes after fertilization. Eggs exposed to low temperatures showed a susceptibility period similar to the resistant period for heat. The resistant period for cold comes some minutes before the first cleavage. Throughout Lyon's work, the measure of susceptibility was the percentage of development of eggs.

Mathews (1906), using Lyon's data and his own slides of sectioned *Arbacia* eggs, attempted to correlate the functional and structural conditions thus presented. From this comparison he concluded that the resistant period which Lyon claimed is just before cleavage really occurs after cleavage, and that immediately before and during cleavage there is, instead, a high susceptibility. Complete justification for making such a comparison and interpretation is doubtful.

A valid comparison would seem possible only if eggs from the same lots as those used by Lyon had been studied.

Mathews thought that the period of greatest susceptibility coincides with the development of the asters and that the period of greatest immunity coincides with the retrogression of the asters and the reconstruction of the nucleus. He suggests that cyanide prevents a nuclear oxidase from reacting upon the cytoplasm, or that it inactivates the active centriole without affecting the inactive centriole substance. There seems to be, however, no direct evidence to support such an interpretation.

Spaulding (1904), using $\frac{1}{64}$ saturated ether solution, found that during the first 12 minutes after fertilization the resistance of *Arbacia* eggs remains the same, but that from this point it gradually rises up to near the beginning of cleavage. There then follows a period of great susceptibility. Spaulding's criterion of susceptibility was the percentage of development.

Moore (1915) studied the rhythmical susceptibility of fertilized sea-urchin eggs to hypertonic sea water and found a maximum just after fertilization and immediately before each cytoplasmic division. Maximal resistance occurs 35–45 minutes after fertilization and just before each division. Moore also used the percentage of development as the measure of susceptibility.

Lillie (1916) studied the action of hypotonic sea water, using the percentage of cytolysis after definite periods of exposure as the criterion of susceptibility. His results also indicate a decline in resistance about the time of formation of the cleavage furrow. He considers this due to a change in the permeability of the plasma membrane. Lillie found no period of increased susceptibility immediately after fertilization and remarks that the membrane change accompanying cleavage is of a different kind from that immediately following fertilization. "The resistance to hypotony does not necessarily depend upon the same conditions as the resistance to cytolytic substances."

Baldwin (1920) exposed *Arbacia* eggs to various higher alcohols and found a well-marked period of susceptibility immediately following fertilization. The resistance then gradually rises until just before cleavage, when a very susceptible period occurs at the exact time of cleavage. Later, the resistant phase reappears until about the time of the second cleavage. The percentage of development was used as a measure of susceptibility.

Just (1922), using the eggs of *Echinarachnius parma*, found that the susceptible period of these eggs to dilute sea water occurs some time before cleavage and while the eggs are still spherical. At the time of cleavage, or when the eggs are beginning to elongate, they are again resistant. Exposing *Arbacia* eggs to dilute sea water, Just (1928*a*) finds two periods of susceptibility, the first occurring during the "pause" (about 35 minutes after fertilization) and the second just before the elongation which precedes cleavage. At the time of cleavage the resistance again returns. The measure of resistance and susceptibility was the rate of cytolysis. Just points out that the rate of cytolysis is correlated with structural changes in the hyaline-plasma layer.

Page (1929) finds that a peak of resistance of *Arbacia* eggs to cytolysis occurs shortly after fertilization and that, as the time for cleavage approaches, the eggs become progressively more susceptible.

A comparison of these results can best be made by plotting all results on a single graph. The difficulty in making such a comparison, however, lies in the fact that since the work was done in different years, various factors, such as time for first cleavage, maturity of eggs, temperature, etc., might have been variable. In the experiments plotted on this graph, only those which showed a closely similar first-cleavage time were chosen. In the various experiments the time for cell division ranged from 52 to 56 minutes after insemination, the exact time beng indicated by the arrows.

From text figure 1 we see that all of the authors, except Lyon and Just, show a period of marked susceptibility just before cleavage and a rise in resistance immediately after. Lyon's greatest resistance comes immediately before cleavage, while Just finds the greatest susceptibility some minutes before division and a resistant phase immediately before division. Baldwin, Spaulding, Moore, and Lyon find, also, a susceptible period immediately after fertilization; while R. S. Lillie, Just, and Page show a resistant period immediately after fertilization.

R. S. Lillie's (1906) suggestion that the resistance to hypotony does not necessarily depend upon the same conditions as resistance to cytolytic substances might be further applied. The various methods used to measure the degree of susceptibility are not neces-

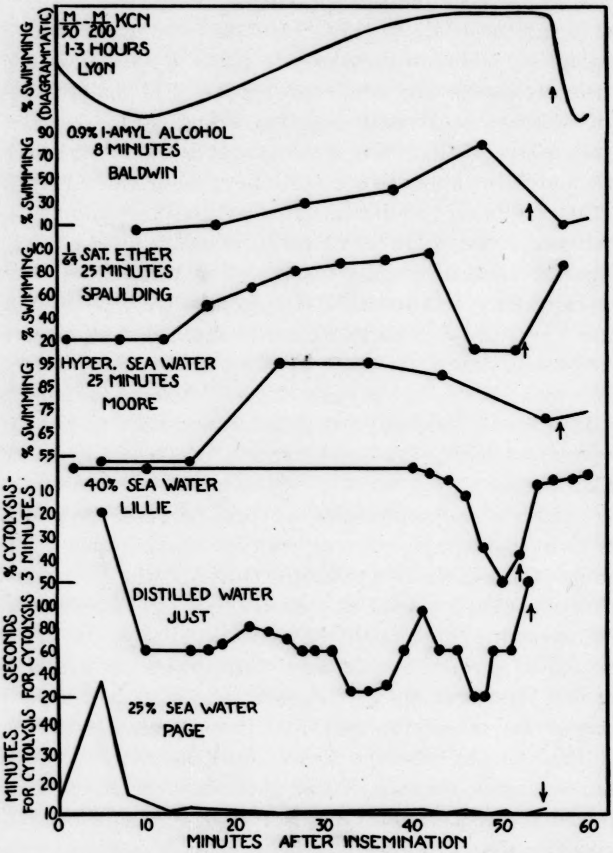


FIG. 1.—Susceptibility rhythms of *Arbacia* eggs to various toxic agents. Ordinates, time for cytolysis or percentage swimming; abscissas, minutes after insemination at which the eggs were exposed. The investigators, agents used, and lengths of exposures are indicated for each curve. The arrows indicate the time for first cleavage. For explanation, see text.

sarily measuring the same kind of susceptibility. Cytolysis and the inhibition of development are not highly comparable processes; and solutions which affect development in a rhythmic manner need not produce a rhythmic rate of cytolysis, or vice versa.

Just suggested that cytolysis is correlated with structural changes in the hyaline-plasma layer of the egg; while Lillie remarks that cytolysis is correlated with the extensibility, and therefore the permeability, of the egg membrane. These two suggestions and that of Mathews are the only attempts made to explain the phenomenon of the observed rhythmic susceptibility periods.

Susceptibility effects, measured by the percentage of development, become evident only after a long period. Such methods, however, give no indication as to whether the eggs really were susceptible at the time when they were actually exposed to the toxic agents, since the readings have to be taken some 10 or more hours after the exposures are made. It is conceivable that the toxic agent might penetrate into the interior of the egg and have no immediate effect on it. Thus, upon transferring the egg to sea water, the toxic agent would remain in the egg and affect it only after certain structures had been developed, which structures are then susceptible.

With the exception of the experiments by Just, the workers already mentioned permitted the eggs to remain in the various solutions for periods varying from 3 minutes to several hours. Under such conditions it is assumed that the eggs stopped development as soon as they were placed in these solutions; otherwise it would be impossible to say at exactly what stage the eggs were being affected. As a matter of fact, such an assumption is entirely untrue, as the present paper will show. The existence of a definite rhythm of susceptibility during the cleavage cycle of *Arbacia* eggs is therefore far from being established.

STATEMENT OF THE PROBLEM

Heilbrunn (1920) mentioned the fact that in dilute KCN the mitotic process of *Arbacia* eggs continues until the spindle is formed, and then stops. This development in KCN was also reported by the author (Blumenthal, 1927*a*, 1928) and by Just (1928*b*) working independently. The present paper deals more fully with the amount and kind of internal development which goes on in the presence of KCN and other anesthetics.

ACKNOWLEDGMENTS

The experiments were performed at the Marine Biological Laboratory during the summers of 1928 and 1929. Appreciation is expressed to Dr. C. E. McClung and Dr. J. H. Bodine for their helpful suggestions and constructive criticism.

METHOD AND RESULTS

Eggs from a single *Arbacia punctulata* were separated into several finger bowls of sea water, care being taken that the eggs were not greatly crowded. The eggs were fertilized and transferred at definite intervals to glass-covered saltcellars containing solutions of KCN, in sea water, in concentrations varying from N/1,000 to N/10. The condition of the eggs before and during exposures was carefully observed. It was found that eggs exposed to KCN, up to within 10–15 minutes of the time when first cleavage is due, will not cleave while the eggs are in the KCN solutions. However, eggs placed in the KCN solutions within 10–15 minutes before cleavage is due will divide in these solutions; yet N/10–N/1,000 KCN solutions are known to act like anesthetics in inhibiting cell division (Blumenthal, 1927*b*).

It is to be noted from Table I that all eggs up to and including those 40 minutes after fertilization at the time of placing in KCN showed no cell division at the time when control eggs were dividing. Eggs 50 minutes after fertilization were in the "dumb-bell" stage when placed in KCN; but at 58 minutes, 50 per cent of these had divided. This was the same time and the same percentage as for the control eggs. At 87 minutes after insemination, or when 50 per cent of the control eggs were in the four-cell stage, these eggs which had already undergone first cleavage in the KCN still remained in the two-cell stage, although the number in this stage had increased to 95 per cent. Eggs 65 minutes after fertilization were practically all in the two-cell stage when placed in the KCN, and at 87 minutes still were in the same stage. Eggs 80 minutes after insemination were all in the two-cell stage before transfer to KCN, but at 87 minutes developed to the four-cell stage, the same as control eggs. Table I also shows that some development in KCN had continued, no matter in what stage the eggs were at the time of exposure. This phenomenon is discussed more fully in a later section of this paper. Table I shows the results of a typical experiment, although about fifty were carried out with various concentrations of KCN. In every case the results were the same.

TABLE I

N/600 KCN. 7/15/28. 2:38 P.M. Air temperature, 24.5° C.
Control: first cleavage = 58 minutes; second cleavage = 87 minutes.

Minutes after Fertilization Placed in KCN	Condition of Eggs before Exposure	Condition of Eggs 58 Minutes after Fertilization and while in KCN	Condition of Eggs 87 Minutes after Fertilization and while in KCN
3......	Fertilization membrane	Monaster	Same as at 58 minutes
10......	Monaster	Small diaster	Same
20......	Small diaster	Large diaster	Same
30......	"Pause"	Streak	Same
40......	Streak	"Dumb-bell"	Same
50......	"Dumb-bell"	50 per cent 2-cell	95 per cent 2-cell
65......	95 per cent 2-cell	....	95 per cent 2-cell
80......	95 per cent 2-cell	....	70 per cent 4-cell

CLEAVAGE IN VARIOUS ANESTHETICS

The same procedure was repeated using various anesthetics, and these in the concentrations found by Lillie (1914) to give an optimum condition of anesthesia of *Arbacia* eggs, which were: 0.25 per cent ethyl nitrate, 10.0 per cent methyl alcohol, 0.04 per cent capryl alcohol, 1.50 per cent ethyl acetate, 0.80 per cent *i*-amyl alcohol, 3.00 per cent *n*-propyl alcohol, 3.00 per cent *i*-propyl alcohol, and 5.00 per cent methyl cyanide.

Except for the methyl cyanide, all these anesthetics produced the same results as the KCN. Eggs placed in these solutions up to 10–15 minutes before the first cleavage is due will not cleave in these solutions. Eggs placed in the anesthetics within 10–15 minutes before cleavage will cleave at the same time and in the same percentage as the control eggs. If cleavage occurs, only the first is completed, the others being inhibited as long as the eggs remain in the anesthetics. The same phenomenon, undoubtedly, occurs during each cleavage cycle, although only the first three could be followed on account of the divergence in cleavage time with increase in the number of cells. Table II shows the results of a typical experiment.

Methyl cyanide, which proved to be the only exception, prevented cleavage at all times even if the eggs were placed in this solution 1 minute before cleavage was due.

TABLE II

N/600 KCN 7/23/28. Air temperature 25° C. 2:58 P.M.
Control: first cleavage = 58 minutes; second = 87 minutes.

Minutes after Fertilization Placed in Anesthetic	Condition of Eggs before Exposure	0.8% *I*-amyl Alcohol	0.04% Capryl Alcohol	3% *I*-propyl Alcohol	3% *N*-propyl Alcohol	0.25% Ethyl Nitrate	1.5% Ethyl Acetate	10% Methyl Alcohol	5% Methyl Cyanide
		Condition of Eggs 58 Minutes after Fertilization and while in Anesthetic*							
10	Monaster	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
20	Diaster	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
30	"Pause"	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
40	Streak	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
46	Beginning metaphase	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
51	"Dumb-bell"	C.	C.	C.	C.	C.	C.	C.	N.C.
55	Beginning elongation	C.	C.	C.	C.	C.	C.	C.	N.C.
		Condition of Eggs 87 Minutes after Fertilization							
68	All 2-cell	2 C.	2 C.	2 C.	2 C.	2 C.	2 C.	2 C.	2 C.
81	All 2-cell	4 C.	4 C.	4 C.	4 C.	4 C.	4 C.	4 C.	2 C.

* N.C. = no cleavage; C. = cleavage; 2 C. = 2-cell; 4 C. = 4-cell.

An attempt was made to get a quantitative comparison between the methods of measuring susceptibility used by previous workers. Eggs which had been exposed to various concentrations of KCN, at definite periods after fertilization, were removed after having been in the KCN for 10 minutes, and placed in normal sea water. An effort was made to determine the subsequent percentage of swimming embryos. It is comparatively easy to determine whether all are swimming or all are dead, but to estimate intermediate proportions correctly is exceedingly difficult. Out of a series of fifteen experiments, only a few gave consistent results.

MITOTIC ACTIVITY IN KCN—CYTOLOGICAL STUDIES

The foregoing experiments were done in 1928. The following year they were repeated and extended, and the eggs involved were studied cytologically in order to observe the effects of KCN at different stages of development. Fertilized *Arbacia* eggs were transferred to N/400 KCN at various periods after insemination and permitted to remain for 10 minutes. They were fixed in Lillie's modified Fleming's, Bouin's fluid, or sublimate-acetic, then sectioned and stained with iron-haematoxylin. For the fixing fluids, Lillie's Fleming gave the best results. Similar experiments were performed with methyl cyanide.

It was found that fertilized *Arbacia* eggs will continue at least 10 minutes of karyokinetic activity in KCN at all stages of development. The rate of this limited mitotic activity is exactly the same as in corresponding control eggs in normal sea water. Plate I shows photographs of sectioned and stained *Arbacia* eggs which had been exposed to N/400 KCN for 10 minutes at various periods after insemination. Normal eggs were also fixed at the instant when eggs were transferred to the KCN and at the time of their removal from the cyanide.

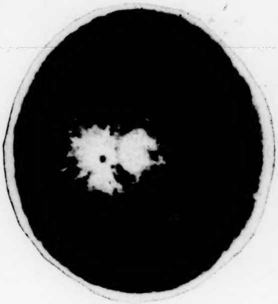
Plate I, Figure 1, shows an egg placed in KCN 2 minutes after fertilization. Normal eggs at this time show the sperm head just inside the egg. Figure 2 is an egg placed in cyanide 4 minutes after fertilization, and shows that development has proceeded as far as the fusion of the pronuclei. Normal eggs at 4 minutes show the sperm advancing toward the egg nucleus but not fused with it. Fusion of the pronuclei in normal eggs occurs at 12 minutes. Figure 3 is an egg in KCN at 6 minutes and shows the growth of the nucleus and aster. At 6 minutes normal eggs show the sperm still closer to the egg nucleus but not yet fused. Figure 4 is an egg in cyanide 8 minutes after insemination; the aster is just beginning to divide. Control eggs show a similar state at about 18–20 minutes, while at 8 minutes they merely show the sperm lying alongside the egg nucleus, corresponding, nearly, to Figure 1. Figure 5 is an egg

PLATE I

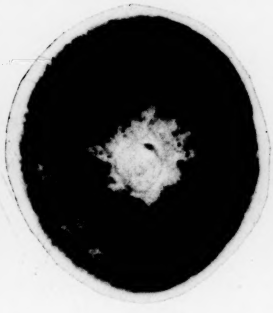
All eggs shown in the photomicrographs were fixed after remaining in N/400 KCN for 10 minutes.

1, Eggs placed in KCN 2 minutes after insemination.
2, 4 minutes after insemination.
3, 6 minutes.
4, 8 minutes.
5, 10 minutes.
6, 15 minutes.
7, 20 minutes.
8, 30 minutes.
9, 35 minutes.
10, 40 minutes.
11, 45 minutes.

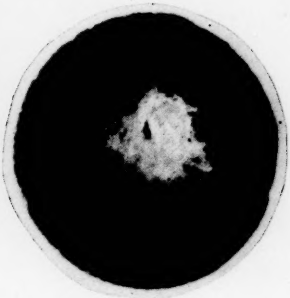
PLATE I



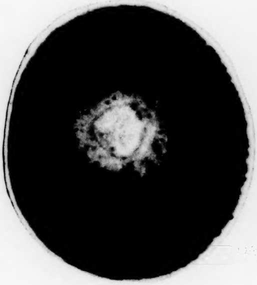
1



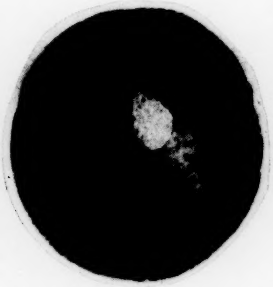
2



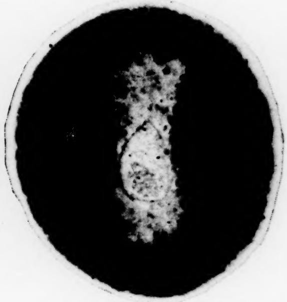
3



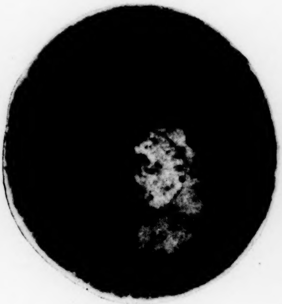
4



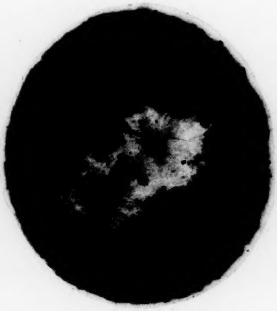
5



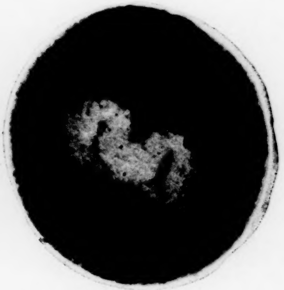
6



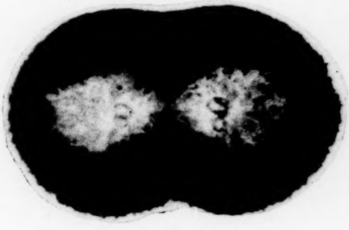
7



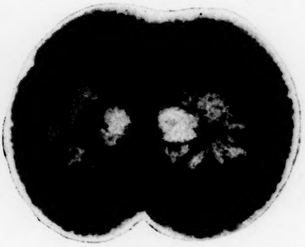
8



9



10



11

placed in KCN 10 minutes after insemination, and shows quite clearly the diaster. Normal eggs at 10 minutes are almost at the stage shown in Figure 2. Figure 6 is an egg in cyanide at 15 minutes after fertilization. The nucleus and asters have increased in size. Normal eggs at 15 minutes are in the stage shown in Figure 3. Figure 7 is an egg put in KCN 20 minutes after fertilization, and shows the large nucleus just before prophase, a stage which coincides with a normal egg about 31–32 minutes after insemination. At 20 minutes normal eggs have just reached the diaster stage. Figure 8 is an egg placed in cyanide 30 minutes after fertilization, and this stage is equivalent to that of normal eggs about 40 minutes after insemination. Normal eggs at 30 minutes simply show a growing diaster. Figure 9 represents an egg placed in KCN at 35 minutes, and corresponds to a normal egg of 45 minutes. At 35 minutes normal eggs are just entering the metaphase stage. Figure 10 shows an egg put in cyanide 40 minutes after insemination, and shows the elongation of the egg preparatory to first cleavage. The daughter nuclei show a vesicular structure. This egg corresponds to a normal egg of 50–51 minutes. Normal eggs at 40 minutes are in the early anaphase or late metaphase. Figure 11 shows an egg placed in cyanide at 45 minutes, or when the eggs were in the telophase stage, and shows cleavage of the egg in the cyanide.

All the eggs shown in Plate I, together with their control eggs, came from a single *Arbacia*. Normal first cleavage (50 per cent) occurred at 53–54 minutes. The photographs show clearly that mitosis has continued in the KCN for at least 10 minutes (since the eggs were removed from the cyanide after 10 minutes) no matter at what stage they were at the time of exposure. It is difficult to determine the exact amount of development (during the early stages) which can continue in KCN since the maturity of the eggs and the temperature varied. From the data available at different temperatures, the amount of later development (from metaphase onward) ranged from 8 minutes at higher temperatures and when control eggs were cleaving at about 45 minutes, to 16 minutes at lower temperatures and when control eggs were cleaving at about 65–68 minutes.

From Plate I, the apparent differences between Figures 6 and 7 are very slight. It might therefore be thought that eggs placed in KCN at 20 minutes after insemination had not developed any farther than those placed in the cyanide at 15 minutes after fertilization, during the 10 minutes in cyanide. As a matter of fact, normal eggs at about 30 minutes after fertilization are in the "pause" period, during which the only sign of development is the increase in the size of the nucleus. In order to prove that such development had actually occurred, the area of over a hundred nuclei of the 15- and 20-minute eggs were measured. Camera-lucida drawings of the nuclei were made on paper and cut out and weighed. Of the 15-minute eggs, 121 nuclei weighed 2.271 grams, or 0.0205 grams per nucleus. Of the 20-minute eggs, 111 nuclei weighed 1.929 grams, or 0.0159 grams per nucleus. The difference between the two is quite significant. Another indication of the greater development of the 20-minute eggs is furnished by the shape of the nuclei. In the 15-minute eggs the nuclei are more nearly round, or oval; while the 20-minute eggs show most of the nuclei elongated preparatory to the breaking of the nuclear membrane.

Heilbrunn (1920) observed that *Arbacia* eggs placed in 0.0025 per cent KCN at 5 minutes after fertilization developed as far as the spindle formation. He also found that eggs placed in 0.004 per cent KCN 12 minutes before cell division was due, cleaved in the cyanide at the same time that control eggs did. The data already presented in this paper confirm Heilbrunn's observations.

The cytological picture of the eggs which had been exposed to KCN seems to be similar to that of normal eggs except for the outermost layer. As seems to be indicated from Figures 2, 3, and 6 of Plate I, the plasma layer is more heavily stained than the inner protoplasm. This might be an indication of a difference in viscosity.

MITOTIC ACTIVITY IN CH_3CN

The action of methyl cyanide is entirely different from that of potassium cyanide. It stops karyokinetic activity at once, no matter at what stage the eggs may be. The cytological picture is also different: all asters fade and disappear within 2 minutes. If the eggs are in metaphase or later, not only do the asters fade out, but the chromosomes appear to be clumped into a small mass. Such eggs, however, will divide if they are returned to sea water.

In a former paper (Blumenthal, 1928) it was shown that eggs placed in methyl cyanide 3 minutes after insemination showed no delay in time for cell division upon return to sea water. Text figure 2 shows that a delay may occur but only under certain conditions. In these experiments, the eggs were placed in the cyanide at various periods after insemination, and permitted to remain for 3 minutes. They were then washed and replaced in sea water, and the time for first cleavage noted. The data shown in this graph agree with the cytological evidence noted above; i.e., no delay in cell division occurs unless the eggs are exposed to the methyl cyanide 35 or more minutes after fertilization. At this time they are in metaphase or later, and, as pointed out above, have the chromosomes clumped together. The closer the eggs are to cleavage at the time of exposure, the longer is the time required for cell division upon return to normal sea water. In text figure 2 are shown the data for two separate experiments performed on two different days. Since the cleavage time for these two sets of experiments was not the same, the data have been recalculated, using 55 minutes as the time for cell division.

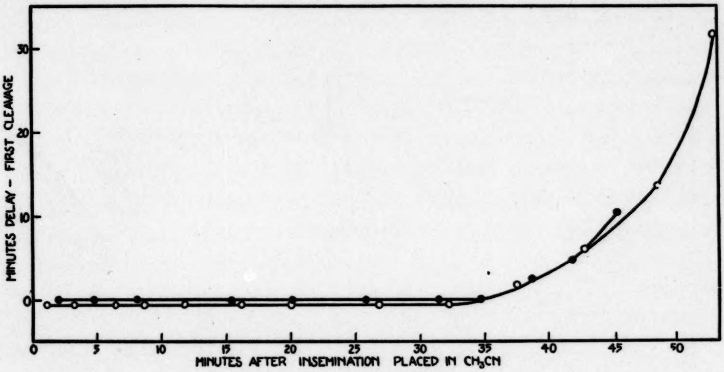


FIG. 2.—Curves show the delay in first cleavage after 3-minute exposures to 5 per cent methyl cyanide. Ordinates are minutes delay; abscissas show the time after insemination at which the eggs were placed in CH_3CN. Two separate experiments are shown, the data having been recalculated with 55 minutes as the time for first cleavage.

EFFECTS OF KCN ON THE CLEAVAGE TIME

In earlier experiments, it was thought that the time required for first cleavage of eggs exposed to KCN at various periods after insemination would be an indication of their rhythms of susceptibility. At that time I had no idea that the eggs could cleave in the cyanide. Under the latter conditions, it can be readily seen that the time for cell division would not be a true index of susceptibility, especially if the eggs were exposed within 10–15 minutes of cleavage. Such experiments, however, might give an indication of the susceptibility of the mechanism concerned in cell division (cytokinesis) to KCN in contrast to that of karyokinesis.

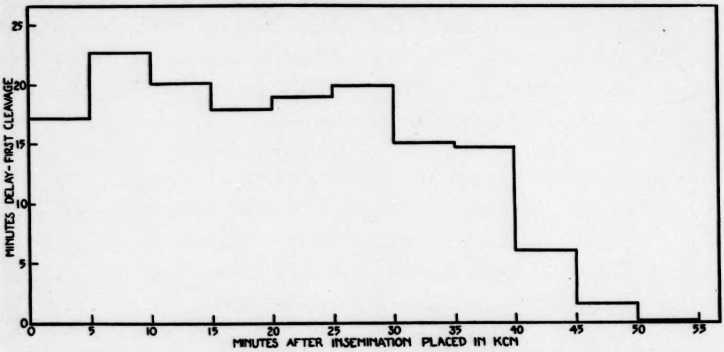


FIG. 3.—Curve shows the delay in first cleavage of eggs exposed to N/400 KCN for 3 minutes at different periods after insemination. This curve is the average of fifteen experiments, all recalculated with 55 minutes as the time for cell division. The data have been plotted as averages over 5-minute periods.

In experiments to demonstrate this, fertilized eggs were exposed to N/400 KCN at various intervals after insemination and, after 3 minutes, removed and washed by the following method: One drop of eggs was placed in a test tube filled with sea water, and the eggs permitted to settle to the bottom. Some of the eggs were then pipetted into a salt-cellar filled with sea water, and the time required for first cleavage noted. The results of such experiments are shown in text figure 3. This curve is the average of fifteen experiments, all recalculated with 55 minutes as the time for cleavage. The abscissas are minutes after insemination at which the eggs were exposed to the KCN; and the ordinates show the delay in cleavage, in minutes. The data have been plotted as averages over 5-minute periods. The graph shows that, in so far as the mechanism of cleavage is concerned, the resistance to KCN increases as the egg approaches division. It must be pointed out, however, that, even though there is no delay in time of the first cleavage, the second is delayed.

MEASUREMENTS OF AMOUNTS OF MITOTIC ACTIVITY

It is well known that development of fertilized eggs is accompanied by many physiological changes. Lillie (1916) pointed out the changes which occur in the properties of the cell membrane in developing *Arbacia* eggs. Heilbrunn (1921) showed the changes in viscosity during mitosis. One could therefore tell when development ceases if he could determine just when these changes fail to occur. The changes in the properties of the cell membrane can be easily followed by determining the rate of cytolysis in dilute sea water. It has already been shown that mitosis will continue for at least 10 minutes in the presence of KCN. If this mitotic development is accompanied by changes in the cell membrane, the time for cytolysis during this development should vary at first, and then remain constant as soon as development ceases. This has been found to be the case.

Eggs were placed in N/600 KCN immediately after fertilization, and transferred to 40 per cent sea water at 1-minute intervals. The time required for cytolysis was recorded. It was found that up to 14 minutes after fertilization the incidence of cytolysis varied from 3 to 5 minutes, but that from this time on it always occurred in 100–110 seconds. The period during which the rate of cytolysis is slow and fluctuating coincides with that during which the development in KCN is going on. As soon as the monaster stage is reached and development ceases, the time for cytolysis falls and remains constant.

The same thing can be shown by still another method. Eggs were placed in N/400 KCN immediately after insemination and then transferred to sea water at short intervals. The time for first cleavage was noted. Up to 15 minutes it fluctuates, but after this time it becomes constant. This appears in text figure 4. Two separate experiments are shown, both having been recalculated with 55 minutes as the time for first cleavage of normal eggs. The time when cleavage becomes constant coincides with that at which development in cyanide stops, as shown by sectioned eggs.

EFFECTS OF LENGTH OF EXPOSURE

In a former paper (Blumenthal, 1928), it was pointed out that the delay in cleavage of fertilized eggs exposed to KCN is independent of the length of exposure but dependent upon the concentration. The same thing is true for the recovery period of unfertilized eggs.

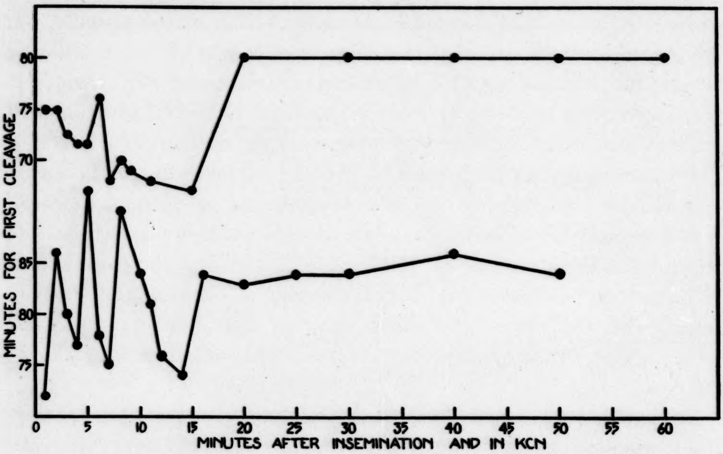


FIG. 4.—Curves show the fluctuation in cleavage time of fertilized eggs removed from N/400 KCN at short intervals during the period when mitosis is continuing in the cyanide. Ordinates are times for first cleavage; abscissas, minutes after insemination and time in KCN. Two separate experiments recalculated with 55 minutes as the cleavage time.

Unfertilized eggs were placed in N/400 KCN and removed after 10 and 30 minutes respectively. The eggs were then washed and inseminated at 2-minute intervals, and the time for cell division observed. Text figure 5 shows the results of these experiments. As the cyanide diffused out of the eggs (after insemination and while in sea water), the delay in cleavage time became shorter, until finally no delay whatever was evident. No significant difference was seen between eggs exposed for 10 minutes and those exposed for 30 minutes.

EXPOSURE TO KCN BEFORE AND AFTER INSEMINATION

In looking for a possible explanation of the fact that a limited amount of karyokinetic activity can occur in an environment of lack of oxygen (KCN), several ideas came to mind. First, it may take 10 or more minutes for the cyanide to get into the egg. Such a possibility, however, is ruled out by the fact that fertilized eggs exposed to KCN for only 30 seconds show a subsequent delay in cell division. Second, the KCN entering the egg during the first 10 minutes is being oxidized according to the idea of Ellinger (1924). Concerning this idea, I have no information. Third, perhaps KCN prevents any external oxygen from entering the cell or forming oxygen compounds within the cell, but that any oxygen already in the cell is utilized. As soon as this oxygen is used up, and since no more is available, mitosis stops. Upon being returned to sea water, the attempt of the egg to rebuild its oxygen reserve might account for the delay in cell division which occurs.

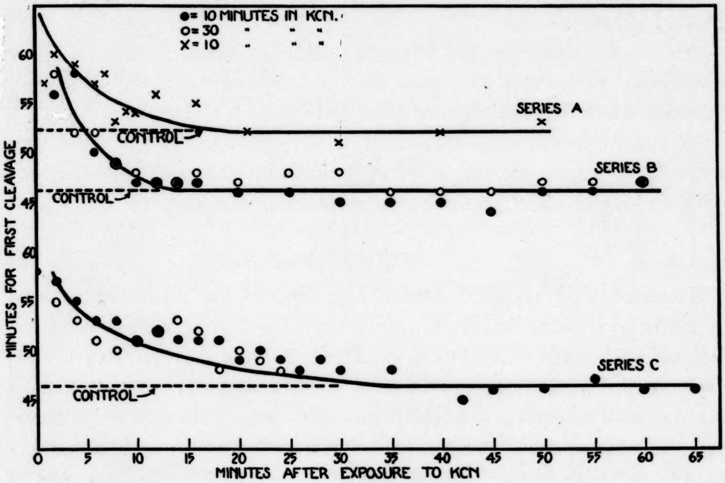


FIG. 5.—Curves show the recovery time of unfertilized eggs exposed to N/400 KCN for 10 and 30 minutes. Data indicate the independence of recovery to length of exposure. Three separate experiments are shown.

Such an idea, however, does not seem tenable in the light of further experiments. Unfertilized eggs were placed in KCN for 60 minutes, at the end of which time we would expect all or most of the internal oxygen to have been used up. If oxygen is needed for the continuation of mitosis, we would expect to find no mitotic development if sperm were added directly to the cyanide. As a matter of fact, the eggs continued to develop in the cyanide in spite of the fact that they were in the KCN continuously for 75 minutes—60 minutes before insemination and 15 minutes immediately after. The cytological picture of such eggs is exactly like that shown in Plate I, Figure 1.

An experiment by Heilbrunn (1920) presents a similar phenomenon. He placed fertilized eggs in 0.004 per cent KCN 5 minutes after insemination and exposed them to a temperature of 10°–12° C. No spindle appeared while the eggs were in the cold; but when they were removed after 2½ hours and the test tube warmed with the hand, spindles soon made their appearance.

DISCUSSION

The ability of fertilized *Arbacia* eggs to continue a limited amount of mitotic activity in KCN indicates the independence between mitosis and oxygen availability. The fact that this activity is limited seems to suggest that there is some secondary effect which is the result of anaërobic metabolism and to which mitosis is susceptible. The cause of this secondary effect may be due to, or comparable to, the increase in the concentration of lactic acid which occurs as the result of anaërobic metabolism in *Arbacia* eggs, as shown by Perlzweig and Barron (1928). Gray (1924) has shown that ciliary activity can continue for 30–45 minutes in the presence of relatively high concentrations of cyanide. Loeb (1902) found that unfertilized sea-urchin eggs could exist for considerable periods of time in cyanide solutions. Amberson (1928) reports that in fertilized *Arbacia* eggs, the oxygen consumption is practically constant between 228 mm. and 20 mm. Hg. partial pressure of oxygen.

The ability of *Arbacia* eggs to continue, at all stages, this limited mitotic activity in KCN is in accord with Gray's (1925) experiments in which he found no marked change in the oxygen consumption of fertilized *Echinus* eggs during their cleavage cycle. This would indicate that no particular stage of mitosis needs more oxygen than any other, so that susceptibility to oxygen deficiency should remain the same throughout the cleavage cycle.

The fact that eggs can develop up to the monaster stage in KCN, even though the eggs have been in the cyanide for 60 minutes immediately preceding insemination, is worthy of attention in view of the experiments by Warburg (1908) in which he found that unfertilized sea-urchin eggs have a definite oxygen consumption which is augmented six- or seven-fold upon fertilization. Similar findings have been reported by Loeb and Wasteneys (1913). According to Loeb (1913), "the observation that the nucleus of the fertilized egg remains unaltered during lack of oxygen or presence of KCN shows that oxidations are the prerequisites of the mechanical processes of nuclear and cell division." Such a conclusion is not in accord with the experiments presented in this paper.

Pantin (1930*a*, 1930*b*, 1930*c*) has demonstrated the ability of *Amoeba* to continue movement in an environment of lack of oxygen and in cyanide. He suggests that amoeboid movement depends directly on anaërobic processes, but that oxygen is required for a recovery process which takes place continuously in the presence of oxygen. The fall in speed of amoeboid movement which occurs during anaërobiosis suggests that adverse effects which inhibit the mechanism of movement are progressively accumulating. The longer the anaërobiosis, the greater is their accumulation, and the longer is the time required for their removal in oxygen by the recovery process. The same sort of phenomenon is observed in the processes involved in muscular contraction. According to Fletcher and Hopkins (1917), the act of contraction is associated with anaërobic reactions; while oxygen is only required for a recovery process. The phenomena observed by Pantin, by Fletcher and Hopkins, and by Gray (ciliary motion) are undoubtedly of the same fundamental nature as mitotic development in KCN.

Mathews (1907) has suggested that mitosis is dependent upon respiratory activity in the cell. According to him, cell division is at its basis a respiratory process. He maintains that astral formation is dependent upon oxygen availability. Such views, however, are not in accord with the present results, where we have found that asters can be formed even in highly concentrated solutions of KCN.

Hammett (1929) has postulated a theory of the mechanism of mitosis in which the sulfhydryl group (−SH) is the essential stimulus of cell division. He states that the $-SH \rightleftharpoons S.S-$ oxidation-reduction system is essential for growth by cell division because the oxygen demand of this process is great and the needs are supplied by this chemical catalytic combination. Such a theory can hardly be reconciled with the findings that mitotic activity can continue in the presence of KCN, where oxidations are entirely inhibited, or very nearly so.

As Lillie (1919) correctly points out, the inference has been drawn from oxidation studies that the same metabolic activities present before fertilization are accelerated, and that this is the real essence of mitosis. However, Loeb and Wasteneys (1912) found that in the starfish there is no measurable increase in oxidation after fertilization, so that the inference drawn above is rather doubtful. Quoting further from Lillie (1919):

> The connection between fertilization and increase in oxidation may thus be incidental. If we realize that the egg is a highly complex system, and that the rate of oxidation as measured is a gross result without any distinction of kind or location, the obvious alternative stands out that we may be measuring quite different metabolic activities before and after fertilization. If this were the case, it is obvious that the net result in terms of oxygen consumption might not change or might show either a gain or a loss, depending on the nature of the system. There may be a retardation of some processes and an acceleration of others, giving a different net result in the sea urchin and starfish, for instance. The mere determination of gain in oxygen consumption, even if it were a universal fact, is not adapted to carry us very far in the problem of fertilization without a more minute analysis.

The evidence presented in this paper more than justifies Lillie's statements.

Heilbrunn (1921) demonstrated viscosity changes during mitosis and found that the formation of the spindle is accompanied by an increase in viscosity. According to him, this activity is possible in KCN because the cyanide acts by intensifying gelation and therefore does not interfere with the increase in viscosity which is necessary for spindle formation. However, the present paper has shown that mitosis in KCN can continue at any stage. Heilbrunn's explanation of the KCN action could not fit the case of those stages of mitosis which are accompanied by a decrease in viscosity and which have been found to continue in cyanide. According to his experiments, he placed fertilized eggs in cyanide 5, 9, 14, and 20 minutes after fertilization and found that development proceeded up to the spindle formation. The 20-minute eggs, according to him, were checked in their development almost immediately. (Normal eggs at 20 minutes already have formed the spindle.) It has already been pointed out that development during the "pause" (20–35 minutes after fertilization) cannot be seen in the living egg, and Heilbrunn studied only living eggs. Sectioned eggs show development during the "pause" accompanied by an increase in the size of the nuclei.

The idea that cytokinesis and karyokinesis are independent of each other is not a new one. Among others, Lillie (1902) has shown that nuclear division can be induced without cytoplasmic division. The present results might be construed to support such a view. We have already seen that the first 10 minutes of mitosis can occur in KCN. If we remove the eggs at some period less than 10 minutes, during which time mitosis has proceeded at the same rate as control eggs, why, then, should there be a delay in cleavage time upon return to sea water? The same thing is seen more strikingly if the eggs are placed in KCN 3 minutes before cleavage is due, and removed to sea water just as cleavage is occurring. Even though the first cleavage occurs at the same time as in control eggs, the second cleavage is delayed. It would seem that KCN produces this delay by its action on the cytoplasm or cell membrane, not having affected the mitotic figure at all.

The possibility that KCN, although diffusing immediately into the cytoplasm, does not penetrate the nuclear membrane for 10 minutes is one which might be considered. This, however, would not explain why the limited mitotic development in KCN continues when the eggs are exposed to the cyanide when no nuclear membrane is present. In the case of methyl cyanide, the breaking-down of the nuclear membrane seems to be of some importance. Although mitotic development is immediately stopped by this cyanide, no subsequent delay in cell division is evident unless the eggs are in metaphase or later at the time of exposure.

It might also seem possible that KCN does not inhibit oxidations or cause a condition of lack of oxygen until 10 minutes after exposure. Such a possibility, however, seems ruled out by the experiments of Barron (1929), who found an almost immediate inhibition of oxygen consumption by *Arbacia* eggs treated with KCN.

Throughout this paper—and, as a matter of fact, in all papers which have to do with KCN experiments—the terms "presence of cyanide" and "lack of oxygen" are used synonymously. For want of evidence to the contrary, and in view of the evidence in support of such an idea, the presence of KCN in concentrations such as have been used in the present experiments may be safely assumed to be equivalent to an atmosphere of lack of oxygen. As a matter of fact, Barron (1929) found that the oxygen consumption of *Arbacia* eggs was entirely suppressed by 0.01M KCN.

SUMMARY AND CONCLUSIONS

1. Fertilized *Arbacia* eggs placed in relatively high concentrations of KCN will continue a limited amount of mitotic activity no matter at what stage they may then be. The amount of development which does occur is at least of 10 minutes' duration.
2. The rate of this limited mitotic activity is exactly the same as in control eggs. In spite of this, cleavage of the eggs upon return to sea water is delayed.
3. Methyl, capryl, propyl, *i*-propyl, *i*-amyl alcohols and ethyl acetate in anesthetic concentrations will not prevent cleavage if the eggs are placed in these solutions at or after metaphase.
4. When cleavage does occur in these solutions and in KCN, the time and percentage cleaved are exactly the same as for control eggs.
5. If one cleavage has occurred and the eggs are still permitted to remain in the anesthetics, no further cleavages take place.
6. Five per cent methyl cyanide stops mitotic activity immediately in all stages. Unlike KCN, methyl cyanide produces a marked effect on the chromosomes and diaster if the eggs are transferred to it during or after metaphase. This condition, however, is readily reversible.

7. Fertilized eggs exposed to methyl cyanide at any stage preceding metaphase show no delay in cleavage time upon their return to sea water. Eggs placed in this cyanide during or after metaphase exhibit a delay in time for cleavage, the delay being greater the closer the eggs are to cleavage at the time of exposure. This phenomenon seems to be in some way related to the breaking-down of the nuclear membrane.

8. Fertilized eggs exposed to KCN at any stage preceding metaphase show a delay in cleavage time upon return to sea water. If the eggs are exposed to KCN after metaphase, almost no delay in cleavage occurs, but the second cleavage is delayed.

9. The cytological picture of eggs which are developing in KCN shows no distinct differences from that of control eggs in the same stages. However, in the KCN eggs, the periphery of the eggs seem to be stained more heavily than the inner cytoplasm.

10. Evidence is deduced to support the idea that karyokinesis and cytokinesis are partly independent processes.

11. Recovery of unfertilized eggs exposed to KCN is independent of the length of exposure but dependent upon the concentration.

12. Evidence is presented to show that mitosis is not directly dependent upon oxygen availability. The inhibition of karyokinesis which finally does occur in KCN may be due to some secondary effect of KCN or of anaërobic metabolism. Similar phenomena have been reported by other workers for amoeboid movement and muscular contraction.

LITERATURE CITED

AMBERSON, W. R. 1928. The influence of oxygen tension upon the respiration of unicellular organisms. Biol. Bull., LV, 79–91.

BALDWIN, F. M. 1920. Susceptible and resistant phases of the dividing sea-urchin egg when subjected to various concentrations of lipoid-soluble substances, especially the higher alcohols. *Ibid.*, XXXVIII, 123–40.

BARRON, E. S. G. 1929. Studies on blood cell metabolism. III. The effect of methylene blue on the oxygen consumption of the eggs of the sea-urchin and starfish. The mechanism of the action of methylene blue on living cells. Jour. Biol. Chem., LXXXI, 445–57.

BLUMENTHAL, R. 1927*a*. Narcosis and cell division in *Arbacia* eggs. Anat. Rec., XXXVII, 160.

———. 1927*b*. The effect of cyanides on the swelling of protoplasm. Biol. Bull., LII, 313–24.

Blumenthal, R. 1928. The effects of certain anesthetics on the cleavage reaction in sea-urchin eggs. Physiol. Zoöl., I, 269–85.

Ellinger, P. 1924. Zur Pharmakologie der Zellatmung. IV. Über den Einfluss der Temperatur auf die Spontanoxydation der Blausaüre an "Brennorten" und auf die Sauerstoffverbrauchskurve von Gewebe bei Cyankalivergiftung. Zeit. physiol. Chem., CXXXVI, 19–29.

Fletcher, W. M., and F. G. **Hopkins**. 1917. The respiratory process in muscle and the nature of muscular contraction. Proc. Roy. Soc., Ser. B, LXXXIX, 444.

Gray, J. 1924. The mechanism of ciliary movement. IV. The relation of ciliary activity to oxygen consumption. *Ibid.*, XCVI, 95.

———. 1925. The mechanism of cell division. II. Oxygen consumption during cleavage. Proc. Camb. Phil. Soc. Biol. Sci., I, 225–36.

Hammett, F. S. 1929. The chemical stimulus essential for growth by increase in cell number. Protoplasma, VII, 297–322.

Heilbrunn, L. V. 1920. An experimental study of cell-division. I. The physical conditions which determine the appearance of the spindle in sea-urchin eggs. Jour. Exp. Zoöl., XXX, 211–37.

———. 1921. Protoplasmic viscosity changes during mitosis. *Ibid.*, XXXIV, 417–47.

Just, E. E. 1922. The effect of dilute sea-water on the fertilized egg of *Echinarachnius parma* during the cleavage cycle. Amer. Jour. Physiol., LXI, 505–15.

———. 1928*a*. Studies of cell division. II. The period of maximum susceptibility to dilute sea water in the cleavage cycle of the fertilized egg of *Arbacia*. Physiol. Zoöl., I, 26–36.

———. 1928*b*. Initiation of development in *Arbacia* eggs. IV. Some cortical reactions as criteria for optimum fertilization capacity and their significance for the physiology of development. Protoplasma, I, 97–126.

Lillie, F. R. 1902. Differentiation without cleavage in the egg of the annelid *Chaetopterus pergamentaceus*. Arch. Entw.-Mech., XIV, 477–99.

———. 1919. Problems of fertilization. Chicago.

Lillie, R. S. 1914. The action of various anaesthetics in suppressing cell division in sea-urchin eggs. Jour. Biol. Chem., XVII, 121–40.

———. 1916. Rhythmical changes in the resistance of dividing sea-urchin eggs to hypotonic sea-water and their physiological significance. Jour. Exp. Zoöl., XXI, 369–402.

Loeb, J. 1902. Über Eireifung, naturlichen Tod und Verlängerung des Lebens beim unbefruchteten Seesterns *Asterias forbesii* und deren Bedeutung für die Theorie der Befruchtung. Arch. Ges. Physiol., XCIII, 59.

———. 1913. Artificial parthenogenesis and fertilization. Chicago.

Loeb, J., and **Wasteneys**. 1912. Die Oxydationsvorgänge um befruchteten und unbefruchteten Seesternei. Arch. Entw.-Mech., XXXV, 535–57.

———. 1913. The influence of hypertonic solutions upon the rate of oxidations in fertilized and unfertilized eggs. Jour. Biol. Chem., XIV, 469–80.

Lyon, E. P. 1902. Effects of KCN and lack of oxygen upon the fertilized eggs and the embryos of the sea-urchin (*Arbacia punctulata*). Amer. Jour. Physiol., VII, 56–75.

———. 1904. Rhythms of susceptibility and of carbon dioxide production in cleavage. *Ibid.*, XI, 52–58.

Mathews, A. P. 1906. The susceptibility of segmenting *Arbacia* eggs and *Asterias* eggs to cyanide. Biol. Bull., XI, 137–40.

———. 1907. A contribution to the chemistry of cell division, maturation and fertilization. Amer. Jour. Physiol., XVIII, 89–111.

Moore, A. R. 1915. On the rhythmical susceptibility of developing sea-urchin eggs to hypertonic sea-water. Biol. Bull., XXVIII, 253–59.

Page, I. H. 1929. Rhythmic susceptibility changes in the cleaving *Arbacia* egg. Brit. Jour. Exp. Biol., VI, 219–28.

Pantin, C. F. A. 1930*a*. On the physiology of amoeboid movement. V. Anaërobic movement. Proc. Roy. Soc., Ser. B, CV, 538–55.

———. 1930*b*. On the physiology of amoeboid movement. VI. The action of oxygen. *Ibid.*, pp. 555–64.

———. 1930*c*. On the physiology of amoeboid movement. VII. The action of anaesthetics. *Ibid.*, pp. 565–79.

Perlzweig, W. A., and E. S. G. Barron. 1928. Lactic acid and carbohydrate in sea-urchin eggs under aërobic and anaërobic conditions. Jour. Biol. Chem., LXXIX, 19–26.

Spaulding, E. G. 1904. The rhythm of immunity and susceptibility of fertilized sea-urchin eggs to ether, to HCl and to some salts. Biol. Bull., VI, 224–40.

Warburg, O. 1908. Beobachtung über die Oxydationsprozesse in Seeigelei. Zeit. Physiol. Chem., LXVII, 6.

INDEX TO VOLUME III

Adrenalectomy, effect of, on polycythemia in white rats, 494

Adrenalin, effect of daily injections of, upon numbers of erythrocytes in the blood of normal and adrenalectomized rats, 494

ALLEE, W. C.; TORVIK, M. M.; LAHR, J. P.; and HOLLISTER, P. L., Influence of soil reaction on earthworms, 164

Amblystoma tigrinum, metamorphosis of, following lung extirpation, 373

ANDERSON, B. G., and BROWN, L. A., Study of chitin secretion in *Daphnia magna*, 485

ANDREWS, E. A.: Honeydew reflexes, 467; evidence from aphids, 467; evidence from membracids, 469; evidences from the action of certain flies, 477; evidence from certain coccids, 478

Ants, honeydew reflexes of, 467

Aphids, evidence from, for honeydew reflexes, 467

Arbacia: the action of ultra-violet rays on egg protoplasm, 330; the effect of intracellular cyanide on cleavage of eggs of, 366

Arbacia punctulata, mitotic development of eggs of, in cyanides, 539

Axolotl, metamorphosis of, following lung extirpation, 373

BANTA, A. M., and BROWN, L. A., Control of sex in Cladocera. VI. By treatment with drugs, 48

BANTA, A. M., and OBRESHKOVE, V., Study of the rate of oxygen consumption of different Cladocera clones derived originally from a single mother, 1

BEYER, KATHE MARGARETE, and CHILD, C. M., Reconstitution of lateral pieces of *Planaria dorotocephala* and *Planaria maculata*, 342

BLUMENTHAL, REUBEN, Mitotic development of *Arbacia* eggs in cyanides, 539

BRINLEY, FLOYD J.: The effect of cyanide on the cardiac rhythm of embryos of *Fundulus heteroclitus*, 283; the effect of intracellular cyanide on cleavage of *Arbacia* eggs, 366

BROWN, L. A., and ANDERSON, B. G., A study of chitin secretion in *Daphnia magna*, 485

BROWN, L. A., and BANTA, A. M., Control of sex in Cladocera. VI. By treatment with drugs, 48

Calcium, necessary for stimulating action of ultra-violet rays on *Arbacia* egg protoplasm, 330

Calcium chloride, effect of pure solution of, on oxygen consumption of *Planaria dorotocephala*, 9

Carassius aureus, action of twenty-three octyl alcohols on, and *Diemyctylus viridescens*, 412

Cardiac rhythm of embryos of *Fundulus heteroclitus*, the effect of cyanide on, 283

CHALKLEY, H. W.: On the relation between the resistance to heat and the mechanism of death in *Paramecium*, 425; the effect of changes in hydrogen-ion concentration on the resistance of *Paramecia* to a temperature of 40° C. when exposure is made (A) in balanced saline, 428; (B) in saline with potassium excess, 430; (C) in saline with calcium excess, 431; (D) in saline with sodium excess, 434

Chick embryo, study of origin and differentiation of the suprarenal gland in, by chorio-allantoic grafting, 201

CHILD, C. M., The susceptibility of *Planaria* to potassium cyanide in relation to hydrion concentration and to certain changes in salt content of the medium, 90

CHILD, C. M., and BEYER, KATHE MARGARETE, Reconstitution of lateral pieces of *Planaria dorotocephala* and *Planaria maculata*, 330

Chitin, secretion of, in *Daphnia magna*, 485

Chloroform, a quantitative study of the effect of, on oxygen consumption of the tadpole (*Rana clamitans*), 519

Chorio-allantoic grafting, study of the origin and differentiation of the suprarenal gland in the chick embryo by, 201

Chortophaga viridifasciata, fatty acids in egg of, 512

Cladocera: rate of oxygen consumption of different clones derived from a single mother, 1; control of sex in, 48; oxygen consumption in the developmental stages of, 271

Cleavage, the effect of intracellular cyanide on, of *Arbacia* eggs, 366

Coccids, evidence from certain, for honeydew reflexes, 478

Coe, W. R., Asexual reproduction in nemerteans, 297

Cold, the effect of exposure to, on the embryonic development of the Japanese beetle (*Popillia japonica* Newman), 291

Contractile vacuole, studies on, in *Spirostomum* and *Paramecium*, 56

Cook, Sherburne F., and Sharman, Frances E., The effect of acids and bases on the respiration of tapeworms, 145

Corey, E. L., Fetal and early postnatal responses of rat gonads to pituitary injections, 379

Corpora lutea, the effect of, on normal maintenance of pregnancy in the albino rat, 231

Corpus luteum hormone, methods of extraction of, 135

Cyanide: the effect of, on the cardiac rhythm of embryos of *Fundulus heteroclitus*, 283; the effect of intracellular, on cleavage of *Arbacia* eggs, 366

Cyanides, mitotic development of *Arbacia* eggs in, 539

Dapnia magna, a study of chitin secretion in, 485

Day, Howard Calvin, Studies on the contractile vacuole in *Spirostomum* and *Paramecium*, 56

Death, on the relation between the resistance to heat and the mechanism of, in *Paramecium*, 425

Development: of insects. I. Fatty acids in the grasshopper egg, 503; mitotic, of *Arbacia* eggs in cyanides, 539

Developmental stages of a cladoceran, oxygen consumption in the, 271

Diemyctylus viridescens, action of twenty-three octyl alcohols on *Carassius aureus* and, 412

Dogfish, excitability of optimum motor points on the dorsal and ventral sides of the spinal cord of, 226

Drosophila: the somatic effects of temperature on a homozygous race of, 392; temperature effect on weight of flies, 394; temperature effect on size, 396; temperature effect on hair number, 400; temperature effect on teeth of sex-combs, 403; temperature effect on the immature period, 404

Earthworms, influence of soil reaction on, 164

Eigenbrodt, Harold J.: The somatic effects of temperature on a homozygous race of *Drosophila*, 392; temperature effect on weight of flies, 394; temperature effect on size, 396; temperature effect on hair number, 400; temperature effect on teeth of sex-combs, 403; temperature effect on the immature period, 404

Elhardt, W. P.; Nice, L. B.; and Morris, Mary Elizabeth, Polycythemia in white rats, 494

Embryonic development, the effect of exposure to cold on the, of the Japanese beetle (*Popillia japonica* Newman), 291

Embryos of *Fundulus heteroclitus*, the effect of cyanide on the cardiac rhythm of, 283

Fatty acids in the grasshopper egg, 503

Fetal and early postnatal responses of rat gonads to pituitary injections, 379

Fevold, H. L.; Hisaw, F. L.; and Meyer, R. K., The corpus luteum hormone. II. Methods of extraction, 135

Fish, effect of nascent oxygen on, 441

Flies, evidences from the action of, for honeydew reflexes; 477

Fragmentation and regeneration in nemerteans, 297

Fraps, M., Studies on respiration and glycolysis in *Planaria*. I. Methods and certain basic factors in respiration, 242

Fundulus heteroclitus, the effect of cyanide on the cardiac rhythm of embryos of, 283

Galeus canis Mitchill, excitability of optimum motor points on the dorsal and ventral sides of the spinal cord of, 226

Garber, S. T., Metamorphosis of the axolotl following lung extirpation, 373

Glycolysis, studies on respiration and, in *Planaria*. I. Methods and certain basic factors in respiration, 242

Gonads, fetal and early postnatal responses of rat, to pituitary injections, 379

Grasshopper, fatty acids in the egg of, 503

Haterius, H. O., and **Nelson, W. O.**, An experimental study of ovariectomy and transplantation in the pregnant albino rat, 231

Heat, on the relation between the resistance to, and the mechanism of, death in *Paramecium*, 425

Heilbrunn, L. V., and **Young, R. A.**, The action of ultra-violet rays on *Arbacia* egg protoplasm, 330

Hess, Olga Theodore, The effects of pure solutions of sodium, potassium, and calcium chlorides on the oxygen consumption of *Planaria dorotocephala*, 9

Hisaw, Frederick L.; **Fevold, H. L.**; and **Meyer, R. K.**, The corpus luteum hormone. II. Methods of extraction, 135

Hollister, P. L.; **Allee, W. C.**; **Torvik, M. M.**; and **Lahr, J. P.**, Influence of soil reaction on earthworms, 164

Honeydew reflexes, 467; evidence from aphids, 467; evidence from membracids, 469; evidences from the action of certain flies, 477; evidence from certain coccids, 478

Hubbs, Carl L., The high toxity of nascent oxygen, 441

Hydrogen-ion concentration: effect of, on respiration of tapeworms, 145; influence of, on earthworm distribution, 164; the effect of changes in, on the resistance of *Paramecia* to a temperature of 40° C. when exposure is made (A) in balanced saline, 428; (B) in saline with potassium excess, 430; (C) in saline with calcium excess, 431; (D) in saline with sodium excess, 434

Hyperthyroidism, effect of experimental, on reproductive processes of female albino rats, 461

Insect development. I. Fatty acids in the grasshopper egg, 503

Japanese beetle, the effect of exposure to cold on the embryonic development of, 291

Lahr, J. P.; **Allee, W. C.**; **Torvik, M. M.**; and **Hollister, P. L.**, Influence of soil reaction on earthworms, 164

Lineus, asexual reproduction in, 297

Ludwig, Daniel, The effect of exposure to cold on the embryonic development of the Japanese beetle (*Popillia japonica* Newman), 291

Lung extirpation, metamorphosis of the axolotl following, 373

Macht, David I., Action of twenty-three octyl alcohols on *Carassius aureus* and *Diemyctylus viridescens*, 412

Melanoplus differentialis, fatty acids in egg of, 505

Membracids, evidence from, for honeydew reflexes, 469

Metamorphosis, of the axolotl following lung extirpation, 373

Meyer, R. K.; **Hisaw, F. L.**; and **Fevold, H. L.**, The corpus luteum hormone. II. Methods of extraction, 135

Mitotic development, of *Arbacia* eggs in cyanides, 539

Moina macrocopa, control of male production in, 48

Moniezia trisonophora, effect of acids and bases on respiration of, 145

Morris, Mary Elizabeth; **Nice, L. B.**; and **Elhardt, W. P.**, Polycythemia in white rats, 494

Motility, a study of, and resistance of rat spermatozoa at different levels in the reproductive tract, 309

Narins, Sidney A., A quantitative study of the effect of chloroform on oxygen consumption of the tadpole (*Rana clamitans*), 519

Nelson, W. O., and **Haterius, H. O.**, An experimental study of ovariectomy and transplantation in the pregnant albino rat, 231

Nemerteans, asexual reproduction in, 297

Nice, L. B.; **Morris, Mary Elizabeth**; and **Elhardt, W. P.**, Polycythemia in white rats, 494

Obreshkove, V., Oxygen consumption in the developmental stages of a cladoceran, 271

Obreshkove, V., and Banta, A. M., A study of the rate of oxygen consumption of different Cladocera clones derived originally from a single mother, 1

Octyl alcohols, action of twenty-three, on *Carassius aureus* and *Diemyctylus viridescens*, 412

Ovariectomy, an experimental study of, and transplantation in the pregnant albino rat, 231

Oxygen, the high toxity of nascent, 441

Oxygen consumption: rate of, of different Cladocera clones derived from a single mother, 1; effects of pure solutions of sodium, potassium, and calcium chloride on, in *Planaria dorotocephala*, 9; of *Planaria* and basic environmental factors, 242; in the developmental stages of a Cladoceran, 271; a quantitative study of the effect of chloroform on, of the tadpole (*Rana clamitans*), 519

Paramecium: on the relation between the resistance to heat and the mechanism of death in, 425; the effect of changes in hydrogen-ion concentration on the resistance of, to a temperature of 40° C. when exposure is made (A) in balanced saline, 428; (B) in saline with potassium excess, 430; (C) in saline with calcium excess, 431; (D) in saline with sodium excess, 434

Paramecium caudatum, studies on contractile vacuole in, 63

Permeability of collodion membranes impregnated with a protein or with a lipoid, 72

Pituitary, fetal and early postnatal responses of rat to injections of, 379

Planaria: susceptibility of, to potassium cyanide in relation to hydrion concentration and to certain changes in salt content of the medium, 90; studies on respiration and glycolysis in. I. Methods and certain basic factors in respiration, 242

Planaria dorotocephala: effects of pure solutions of sodium, potassium, and calcium chlorides on the oxygen consumption of, 9; reconstitution of lateral pieces of, 330

Planaria maculata, reconstitution of lateral pieces of, 330

Polycythemia, in white rats, 494

Popillia japonica Newman, the effect of exposure to cold on the embryonic development of, 291

Potassium chloride, effect of pure solutions of, on oxygen consumption of *Planaria dorotocephala*, 9

Potassium cyanide, susceptibility of *Planaria* to, in relation to hydrion concentration and to certain changes in salt content of the medium, 90

Protoplasm, the action of ultra-violet rays on *Arbacia* egg, 330

Rana clamitans, a quantitative study of the effect of chloroform on oxygen consumption of the tadpole of, 519

Rats: fetal and early postnatal responses of gonads of, to pituitary injections, 379; effect of experimental hyperthyroidism on reproductive processes of female albino, 461; polycythemia in white, 494

Reconstitution of lateral pieces of *Planaria dorotocephala* and *Planaria maculata*, 330

Reflexes, honeydew, 467

Regeneration, in nemerteans, 297

Reproduction, asexual, in nemerteans, 297

Reproductive processes, effect of experimental hyperthyroidism on, of female albino rats, 461

Reproductive tract, a study of the motility and resistance of rat spermatozoa at different levels in, 309

Respiration: effect of acids and bases on, in tapeworms, 145; studies on, and glycolysis in *Planaria*. I. Methods and certain basic factors in, 242

Rice, Kenneth S., The permeability of collodion membranes impregnated with a protein or with a lipoid, 72

Rizzolo, Attilio, The excitability of optimum motor points located on the dorsal and ventral sides of the spinal cord of the smooth dogfish (*Galeus canis* Mitchill), 226

Secretion, of chitin in *Daphnia magna*, 485

Sex, control of, in Cladocera, 48

Sharman, Frances E., and Cook, Sherburne F., The effect of acids and bases on the respiration of tapeworms, 145

Simocephalus exspinosus, oxygen consumption in the developmental stages of, 271

Slifer, Eleanor H., Insect development. I. Fatty acids in the grasshopper egg, 503

Sodium chloride, effects of pure solution of, on oxygen consumption of *Planaria dorotocephala*, 9

Soil reaction, influence of, on earthworms, 164

Somatic, effects of temperature on a homozygous race of *Dorsophila*, 392

Spermatozoa, a study of the motility and resistance of rat, at different levels in the reproductive tract, 309

Spirostomum ambiguum, studies on contractile vacuole in, 56

Spirostomum teres, studies on contractile vacuole in, 56

Splenectomy, effect of, on polycythemia in white rats, 494

Suprarenal gland, study of the origin and differentiation of, in the chick embryo by chorio-allantoic grafting, 201

Susceptibility of *Planaria* to potassium cyanide in relation to hydrion concentration and to certain changes in salt content of the medium, 90

Tadpole, a quantitative study of the effect of chloroform on oxygen consumption of, 519

Tapeworms, effect of acids and bases on respiration of, 145

Temperature: the effect of low, on the embryonic development of the Japanese beetle (*Popillia japonica* Newman), 291; the somatic effects of, on a homozygous race of *Drosophila*, 392; effect on weight of flies, 394; effect on size, 396; effect on hair number, 400; effect on teeth of sex-combs, 403; effect on the immature period, 404; the effect of changes in hydrogen-ion concentration on the resistance of *Paramecia* to, of 40° C. when exposure is made (A) in balanced saline, 428; (B) in saline with potassium excess, 430; (C) in saline with calcium excess, 431; (D) in saline with sodium excess, 434; time at which chitin secretion begins in *Daphnia magna* is a function of, 485

Torvik, M. M.; Allee, W. C.; Lahr, J. P.; and Hollister, P. L., Influence of soil reaction on earthworms, 164

Toxicity, high, of nascent oxygen, 441

Transplantation, an experimental study of ovariectomy and, in the pregnant albino rat, 231

Ultra-violet rays, the action of, on *Arbacia* egg protoplasm, 330

Viscosity, of *Arbacia* egg protoplasm increased by action of ultra-violet rays, 330

Weichert, Charles K., Effect of experimental hyperthyroidism on reproductive processes of female albino rats, 461

Willier, B. H., A study of the origin and differentiation of the suprarenal gland in the chick embryo by chorio-allantoic grafting, 201

Yochem, Donald E., A study of the motility and resistance of rat spermatozoa at different levels in the reproductive tract, 309

Young, R. A., and Heilbrunn, L. V., The action of ultra-violet rays on *Arbacia* egg protoplasm, 330

Diet and Efficiency

A FIVE-YEAR CONTROLLED
EXPERIMENT ON MAN

By
HARALD G. O. HOLCK

THE report of an elaborate self-experiment to secure objective evidence of the effects of eating habits. Everything is recorded—food intake, time, blood pressure, basal metabolism, results of muscular endurance and mental tests, and the comparison of these records with similar observations made during a control period.

Paper, $1.10 postpaid

The University of Chicago Press

The University of Chicago Science Series

The purpose of this series is to present the latest results in the various fields of scientific research in as summary a manner and with as little technical detail as is consistent with sound method.

All interested in the most recent progress in science will find in this series the complete results of current investigations given in a compact and attractive form by well-known specialists.

The University of Chicago Press
Chicago - - - - - Illinois

The Epidemiology and Control of Malaria in Palestine

By ISRAEL J. KLIGLER
Professor of Hygiene and Bacteriology at Hebrew University, Jerusalem

This book is the report of how country-wide malaria control was effected in Palestine. The method of studying and coping with the mosquito vector is fully described as one of the greatest factors in the epidemiology of malaria. Dr. Kligler also indicates that measures taken in the Near East can be made to apply to conditions in other localities.

Illustrated with many photographs and diagrams of regions, temperatures, and local anopheles vectors.

Published in March—$5.00

THE UNIVERSITY OF CHICAGO PRESS

A complete and up-to-date summary of progress in fields in which unusually rapid progress has been made during the last decade

The Newer Knowledge of Bacteriology and Immunology

Edited by EDWIN O. JORDAN and I. S. FALK

Written by eighty-two contributors

In making available the latest results of investigation in the various lines of bacteriology and immunology, the editors have sought out the leading men in the field. Each of the eighty-two authors has made a definite contribution to scientific knowledge and his chapter represents his latest conclusions.

Such men as these have contributed: Noguchi of the Rockefeller Institute, Clark of Johns Hopkins University, Landsteiner of the Rockefeller Institute, Zinsser of Harvard, Meyer of the University of California, Winslow of Yale, Wells of the University of Chicago, Rosenau of Harvard, Hegner of Johns Hopkins University, Kolmer of the University of Pennsylvania, Park of the Bureau of Laboratories in New York City, and Breed of the Agricultural Experiment Station in Geneva, New York.

All unnecessary duplication and overlapping has been avoided, but in cases where an actual divergence of opinion exists, this has been made clear to the reader by the inclusion of papers of opposite views.

The whole project has been undertaken as a co-operative enterprise in which science will be the chief beneficiary. By furnishing landmarks of progress, and by affording suggestions on significant problems of current interest, these contributing scientists have provided a new basis for continued research.

$10.00

THE UNIVERSITY OF CHICAGO PRESS
CHICAGO, ILLINOIS